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Light-based Methods for Whole Blood Bacterial Inactivation Enabled by a Recirculating Flow System

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Abstract

Light of certain wavelengths can be used to inactivate pathogens. Whole blood is opaque, thus the penetration of light is reduced. Here, we overcame this limitation by using a thin transparent tube that is illuminated from all angles. Three light-based techniques were evaluated: photodynamic therapy (PDT) using a 660-nm light and antibody-photosensitizer conjugates, ultraviolet, and violet light. We observed a reduction of 55–71% of *Staphylococcus aureus* after 5 h of exposure (starting concentration 10^7 CFU/mL), and an 88–97% reduction in Methicillin-resistant *Staphylococcus aureus* (MRSA) (starting 10^4 CFU/mL). An 83–92% decrease for *S. aureus* and 98–99.9% decrease for MRSA was observed when combined with an immunocapture approach. Complete blood count with differential analysis did not reveal any significant changes in the blood cell numbers. Genotoxicity studies showed that violet and ultraviolet did not induce any significant level of single strand breaks and alkali labile sites in the peripheral blood mononuclear cells (PBMC). In contrast, ultraviolet did induce a very low level of cyclobutane pyrimidine dimers, a UV damage indicator. PDT generated a significant level of single strand breaks and 8-oxoGua in these cells. The approaches showed promise for whole blood pathogen inactivation with minimal collateral damage to PBMC.

Abstract

Light of certain wavelengths can be used to inactivate pathogens. However, whole blood is opaque, thus the penetration of light is reduced. Here, we overcame this limitation by using a thin transparent tube that is illuminated from all angles. Three light-based techniques were evaluated: photodynamic therapy (PDT) using a 660-nm light and antibody-photosensitizer conjugates, ultraviolet, and violet light. We observed a reduction of pathogens after exposure. The approaches showed promise for whole blood pathogen inactivation with minimal collateral damage to PBMC.

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Keywords

Blood safety; transfusion; pathogen inactivation; whole blood; penetration depth of light; PDT; UV; comet assay; DNA damage

Introduction

Transfusion transmitted infectious diseases (TTI) are a growing concern (1, 2). Donated blood is screened for major infectious pathogens (3, 4). This screening along with multiple layers of other safety measures reduce TTI significantly (1, 5). However, only 4–6 pathogens (mostly viruses) are currently screened in most countries, thus several high risk pathogens (such as pathogenic bacteria) remain unscreened (6, 7). The poor sensitivity of current screening technologies (about 50%) for pathogen quantities below the detection limit results in infections even when the blood is screened (6, 8, 9). For instance, transfusion-associated sepsis is a major cause of mortality along with other transfusion-related diseases (5, 6). While it is necessary to test for blood safety, screening every individual blood pack for all possible pathogens is very time consuming and costly. Several pathogen reduction technologies (PRTs) have been developed to address these needs. PRTs inactivate pathogens via photoactivatable reagents such as amotosalen (10), psoralen (11), riboflavin (12, 13), and other photosensitizing agents (14). Most PRTs are non-specific, and are therefore applicable to a variety of organisms. Even when the pathogen concentration is below the detection limit of screening methods, PRTs are still able to eliminate pathogens and thus ensure the safety of blood products. Currently, PRTs have been approved for use in some European countries (10, 12, 13), and are in limited use in the US (14). Examples of PRTs include the INTERCEPT™ (10), the Mirasol® (12, 13), and the THERAFLEX systems (14).

Commercially available PRTs focus on separated blood components such as purified platelets and plasma. A major reason for blood component separation is that the hemoglobin in red blood cells (RBC) absorbs light spectra ranging from UV to a large portion of the visible light region (15). Due to their abundance and absorption characteristics, RBCs obstruct the penetration of light through blood, which limits the applications of PRTs to only platelets and plasma. There is a need to apply PRTs to whole blood (10, 13). To this end, we aimed to photo-inactivate pathogens directly in whole blood by recirculating the blood

through a thin transparent tube, while illuminating the tube with a light source. The tube resides inside a reflective chamber (Fig. 1) and by flowing the blood through this tube, and illuminating from all angles, the exposure to light can be maximized, potentially overcoming the blocking effects of hemoglobin. Feasibility has been demonstrated previously with circulating tumor cells (16), but not for blood-borne infectious organisms in constant flow. In this report, three different wavelengths, 660 nm (red), ultraviolet-C (UV-C), and violet (390–415 nm) were examined. The use of 660 nm was to activate a photosensitizing agent (Chlorin E6), conjugated to an antibody (CE6-Ab) in order to target specific pathogens (*S. aureus* and MRSA), based on the principle of photodynamic immuno-therapy (PIT). UV-C and violet illumination was based on the germicidal capability of light in these wavelengths, without the need for additional photoactivatable reagents. UV light is widely used to kill bacteria and viruses, but not in blood. UV irradiation has also been used in surgical wound disinfection (17, 18). Also, the photo-inactivation principle was combined with removal of pathogen by immunocapture (19) to enhance the pathogen reduction efficiency. Two representative, gram-positive bacteria, *S. aureus* and its antibiotic resistant equivalent, MRSA, were used for this proof-of-concept work.

Our methodologies utilize reactive oxygen species and light irradiation at certain wavelengths, which could potentially cause collateral damage to normal blood cells. Therefore, we also report the evaluation of potential adverse effects to normal blood components using two methods: complete blood count with differential test (CBC differential) and genotoxicity evaluation, employing the Comet assay (single-cell gel electrophoresis).

Materials and Methods

Bacterial culture and whole blood.

S. aureus and MRSA strains were purchased from the American Type Culture Collection (ATCC 12598 for *S. aureus* and ATCC 43300 for MRSA). *S. aureus* was propagated in ATCC Medium 3 (nutrient broth or agar) at 37 °C in a shaking incubator while MRSA was propagated in tryptic soy broth (TSB) or agar (TSA). Bacterial concentrations were determined by both OD-600 value (optical density value at 600 nm), which was measured with a UV-VIS spectrometer (Spectronic 20 Genesys, Spectronic Instrument) in their appropriate broths, and their corresponding colony count from the agar plate. The initial OD-600 value in the range of 0.02–0.04 (about $1\text{--}2 \times 10^7$ CFU/mL for both bacteria) was used. For MRSA the bacterial concentration was further adjusted to $\sim 10^4$ CFU/mL by serial dilutions. These quantities represent excess bacterial load compared to clinically relevant levels in septicemia diagnosis (1–100 CFU/mL (20)) or in contamination found in blood products ($< 10^2\text{--}10^3$ CFU/mL) (21, 22). 0.5 mL of 10x concentrated bacterial culture was added to 4.5 mL of whole blood for a total volume of 5 mL. Human and monkey whole blood with an anticoagulant (sodium citrate) was purchased from vendors such as Innovative Research (Novi, MI) and Worldwide Primates Inc (Miami, FL).

Experimental setup.

A temperature-controlled bath, a peristaltic pump, and an illumination chamber (or two LEDs for illumination) were included in the apparatus (Fig. 2). Whole blood (5 mL) spiked with bacteria was placed in 15 mL sterile culture tubes. The culture tubes were inserted in a water bath set to 37 °C that rested on a small heating stirrer plate. The blood was also agitated with a mini magnetic stirrer (7 mm x 2 mm) inside the solution. Polydimethylsiloxane (PDMS) tubing from Dow Corning (SILASTIC laboratory tubing) having a length of 120 cm was connected to the blood sample. The inner diameter of the tube used in UVC and PDT was 1.02 mm, outer diameter was 2.02 mm, and the thickness was 0.5 mm. For the violet light, the inner diameter of the tube was 0.6 mm, outer diameter was 1.2 mm, and the thickness was 0.3 mm. The tubes ran through a peristaltic pump (P-3, Pharmacia) to maintain a constant flow rate. The remainder of the tubing was placed in the illumination chamber to maximize the light exposure by reflecting it from all sides (in case of violet illumination, top and bottom). The temperature inside the illumination chamber was monitored for several hs to ensure that heat generated by the light sources did not reach temperatures that could cause thermal damage to the cells. The illumination chamber temperature was equilibrated at 29 – 31 °C for all techniques. The tube was connected back to the blood sample to complete the circulation system. The pumping speed of the peristaltic pump was calibrated to a 0.5 mL/min flow rate. The entire apparatus was installed in a biosafety cabinet to prevent contamination. The experimental parameters such as tube size, length, flow rate, and duration were determined by performing preliminary tests. Controls with and without pumping demonstrated that there were negligible differences in bacterial growth, we therefore did not pump the blood through tubes in our controls. Outcomes were evaluated by comparing treated to untreated (control) blood at the end of the 5 h period. Typically, the blood circulated through the tube using a peristaltic pump at a rate of 0.5 mL/min for 5 h. The experiments were repeated 5 times (n=5) for each of the techniques. The light intensity was measured by Newport 843-R Power Meter Kit using 818-UV/DB Sensor (sensor diameter, 10.25 mm, 200–1100 nm, 0.2 W maximum) inside the reflective chamber facing the light source, and the same location that the samples resided.

Photodynamic therapy (PDT) using photosensitizer-antibody conjugates.

Chlorin E6 (CE6, Frontier Scientific, Logan, UT) was chosen because it is a naturally occurring, commercially available photosensitizer that has excitation maxima around 667 nm excitation where the light absorption by hemoglobin is minimal, and has a relatively high quantum efficiency. It has been used for photodynamic inactivation of bacteria and other organisms (23–25). The CE6 molecule has three carboxyl groups which can be used for conjugation with antibody via a process described in our prior publication (16). CE6 was conjugated to (i) a polyclonal antibody to *S. aureus* (PA1–7246, Thermo Fisher Scientific, Waltham, MA) and (ii) Penicillin binding protein 2a (PBP2a) monoclonal antibody for MRSA (26) (10-P08B, Fitzgerald Industries International, Acton, MA). PBP2a overexpression is a known indicator for antibiotic resistance in *S. aureus* (26), and hence can be used to target MRSA. The conjugation reaction was performed at room temperature with agitation. The resulting CE6-Ab conjugates were stored at 4 °C, until use. All conjugates

were used within 1–2 weeks from their preparation. Based on the input, we estimated the concentration of Ce6 in the conjugate is about 0.5 mg/mL.

The bacteria-inoculated blood was prepared in the same way as described in the “Bacterial culture and whole blood” section, except that the CE6-Ab conjugates (200 μ L) were added to the blood. The middle part of the tube was inserted in the illumination chamber, which was made of mirrors (30.5 $\times$ 30.5 $\times$ 30.5 cm) (Fig. 2-a). To allow sufficient time for the CE6-Ab conjugates to bind with target bacteria, the blood mixture was circulated at 0.5 mL/min through the tube without illumination for the first 2 h. Illumination with 660 nm LED light (90 $\times$ 1 Watt LED High Power Round Grow Light (Solid Red model), LED Wholesalers; for spectrum information see Supporting Information, Fig. S3) was then performed for 3 h at a light intensity of 0.355 μ W.

UV-C exposure of tube.

S. aureus and MRSA were spiked into whole blood samples. The blood was circulated through a tube at flow rate of 0.5 mL/min. Part of the tube was inserted into the chamber (Fig. 2-b), which contained a UV-C light (ODYSSEA UVC-18W; see Fig. S3) and illuminated the samples for 5 hs at a light intensity of $\sim$ 1.84 μ W. It has been reported that PDMS is only partly transparent in the UVC spectral region (27). In order to determine the transparency in UVC the transmittance was measured using a UVX Radiometer at a distance similar to the one in the experiments (about 4 inches from the UVC light). Without a tube the irradiance (intensity) was 465 μ W/cm². In the presence of half PDMS tube the intensity dropped to 325 μ W/cm². A 140 μ W/cm² or $\sim$ 30% reduction was observed. Therefore, an estimated 70% (1.28 μ W) of the measured intensity is delivered to the blood.

Violet light exposure of tube.

Bacteria inactivation capabilities of light at 415 nm wavelength have been reported (28, 29). The tube diameter was decreased to 0.64 mm because bacteria inactivation was not observed when a 1.02 mm diameter tube was used (perhaps due to the weaker germicidal effect of violet light). The tube was coiled over a custom built transparent panel as shown in Fig. 2-c. Two violet LED lights (MinionWeb, 30 Watt LED UV Blacklight (405nm); for spectrum information see Fig. S3) were placed on either side of the tube. Unlike the other two techniques, the tube in violet illumination setup was cooled using a fan during the experiment. The temperature around the coiled tube was maintained at $\sim$ 29 $^{\circ}$ C. MRSA (at $\sim$ 10⁴ CFU/mL) was spiked in whole blood for these tests. The blood was circulated at 0.5 mL/min flow rate and illuminated for 5 h at a light intensity of $\sim$ 55.1 μ W. This method was only investigated with MRSA spiked blood.

Enhancing inactivation by immunocapture.

The aforementioned three light based techniques were also combined with immunocapture. The internal surface of a PDMS tube was modified as described in our prior work (19, 30). Antibody (*S. aureus* polyclonal antibody (PA1–7246, Life Technologies)) and Penicillin binding protein 2a (PBP2a) monoclonal antibody for MRSA (10-P08B, Fitzgerald Industries International) were coated on the inner surface of the tube. The blood was circulated through

two tubes: the antibody coated tube without illumination, and the unmodified tube with light illumination simultaneously.

Bacterial growth monitoring by colony counting.

During the pathogen inactivation procedure, 50 μ L of inoculated blood was extracted at the following time points: 0 h, 1 h, 3 h, and 5 h. The *S. aureus* infected blood was diluted in 450 μ L of nutrient broth to generate a 10 x dilution, three additional dilutions were performed (100 x, 1000 x, 10000 x). 10 μ L of each of the diluted samples were streaked on a 5% sheep blood agar plate (Fisher Scientific, Hampton, NH) that was divided into quadrants. The MRSA infected blood (50 μ L) was spread directly onto sheep blood agar plates without additional dilution. The bacteria colonies were allowed to grow overnight and counted using the particle analysis function in ImageJ (National Institutes of Health). The outcomes of pathogen inactivation were analyzed and presented as a percentage reduction [mean $\pm$ standard error of mean (SEM)] using the colony counting results. We used percentages due to batch-to-batch variations in bacterial growth rate (the full set of data is presented in Figures S1 and S2).

Genotoxicity investigations.

As the above light sources can induce DNA damage, such as oxidatively generated 8-oxo-7–8-dihydroguanine (8-oxoGua), and cyclobutane pyrimidine dimers (CPD), the genotoxicity of these techniques was studied using the Comet assay.

Experiments were performed with animal whole blood without bacteria. Monkey blood (cynomolgus) was purchased from Worldwide Primates INC, (Miami, FL). The experiments were repeated as described above. 1 mL aliquots of blood were collected into vacutainer tubes with anticoagulant (EDTA) before the start of the experiments and at the completion of the experiments 5 h later. 250 μ L of blood was immediately stored in a freezer (-20°C) for genotoxicity studies, and the remainder was shipped to Antech Diagnostics for CBC differential test. The studies were repeated three times for each technique.

Enzyme-modified, and alkaline comet assay for whole blood.

The alkaline and enzyme modified comet assay method was performed using Compac-50 HTP electrophoresis kit (Cleaver Scientific, Rugby, UK) as described previously (31, 32) with the following minor modifications:

Briefly, the exposed blood samples (which had been frozen at -20°C) were defrosted at room temperature. 5 μ L of whole blood (33) from each sample was then suspended directly in 80 μ L of 0.6% low melting point agarose gel (Invitrogen, Carlsbad, USA), and then dispensed onto glass microscope slides, pre-coated with 1 % normal melting point agarose. The agarose gels were then allowed to set under a 22×22 mm cover slip by placing the slides on a cold, slide chilling plate (Cleaver Scientific, Rugby, UK). When the gels were set, the cover slips were removed gently and the slides placed vertically in HT vertical racks included in the Compac-50 kit. The HT racks were then placed in the lysis dishes containing ice-cold lysis buffer (100 mM disodium EDTA, 2.5 M NaCl, 10 mM Tris-HCl, pH 10, containing 1 % triton X-100 which was added freshly) overnight. Afterwards, the HT racks

containing the slides were transferred to washing dishes filled with cold, double-distilled water for 30 min.

In order to detect the level of 8-oxoGua or CPD, the relevant gels were incubated with hOGG1 or T4endoV enzymes, respectively, prior to the electrophoresis. Performing comet assay in the absence of T4endoV or hOGG1 at pH >13, allowed for the analysis of strand breaks (SB) and, alkali labile sites (ALS), defined throughout as SB/ALS.

Human 8-oxoguanine glycosylase 1 (hOGG1)-modified comet assay (hOGG1 comet).

To detect oxidized purines, 50 μ L of hOGG1 enzyme (New England Biolabs, Hitchin, UK) diluted in enzyme reaction buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM Na₂EDTA, 0.2 mg/mL BSA, pH 8 adjusted with KOH) was added to each gel (final concentration 1.6 U/mL), and the slides incubated at 37 °C, in a humidified atmosphere, for 45 min (33). The slides were then transferred for electrophoresis and the remainder of the comet assay.

T4 endonuclease V-modified comet assay (T4endoV comet).

CPD were assessed using the T4endoV comet-modified comet assay (32). 60 μ L of T4endoV (0.1 U/ μ L) diluted in enzyme reaction buffer, were added to the gels and the slides were incubated for 60 min, at 37 °C in a humidified atmosphere. The slides were then transferred for electrophoresis and the remainder of the comet assay.

Comet assay electrophoresis.

The HT racks containing the slides were placed in the Compac-50 tank, and the tank was filled with 550 mL of ice cold alkaline electrophoresis buffer (300 mM NaOH, 1 mM disodium EDTA, pH 13) for 20 min and then underwent electrophoresis at 25 V for 20 min. Slides were then neutralized by transferring the HT racks to neutralization dish filled with 0.4 M Tris-HCl, pH 7.5 for 20 min. The slides were then washed again by being submerged in distilled water inside the washing dishes, prior to an overnight drying at room temperature whilst still in the racks. For staining, the slides in the HT rack were submerged in distilled water, prior to being submerged in a freshly made solution of 2.5 μ g/mL propidium iodide for 20 min (31). The HT racks were again submerged in distilled water for 30 min and allowed to drain and dry prior to scoring. All procedures were carried out under subdued light to minimize possible adventitious DNA damage. All slides were then observed and fifty comets per gel selected and scored using a fluorescence microscope (Axioskop; Carl Zeiss, Jena Germany) and Comet Assay IV analysis software (version 4.2; Perceptive Instruments, Haverhill, Suffolk, UK). Analyses of DNA damage were repeated in their entirety on two different occasions using same samples.

Results

PDT of *S. aureus*-inoculated blood yielded an average 71.3 ± 5.2 % decrease in *S. aureus* levels compared to the control values. PDT on MRSA spiked blood resulted in an average 88.3 ± 11.7 % reduction. UV-C exposure resulted in an average 56.9 ± 8.4 % decrease for *S. aureus* and an average 97.4 ± 2.6 % reduction for MRSA. violet illumination provided an average 88.3 ± 3.8 % reduction for MRSA. The combination of these techniques with

immunocapture (abbreviated to CAP) resulted in considerable improvement in pathogen reduction. The PDT + CAP combination resulted in an average 83.7 ± 4.6 % reduction for *S. aureus* and an average 98.2 ± 1.8 % decrease for MRSA. The UV-C + CAP combination resulted in an average 88.7 ± 3.3 % reduction for *S. aureus* and an average 99.9 ± 0.1 % reduction for MRSA. Violet + CAP combination was performed only on MRSA and achieved an average 98.4 ± 1.6 % reduction. The combination of all three PDT + UV-C illumination + CAP (PDT + UV-C + CAP) was performed on blood spiked with *S. aureus*, and resulted in an average 92.0 ± 8.0 % reduction. A summary of these results is presented in Fig. 3 (details for these experiments are presented in Figures S1 and S2) in all these comparisons the percentage reduction is derived by comparing the experimental results to controls at final pathogen concentrations.

The CBC differential analysis showed a statistically significant change ($P < 0.05$) including an increase of the mean corpuscular hemoglobin concentration (MCHC) value, a decrease of platelet count, and an increase in monocyte count in PDT, by comparing blood before and after exposure. These changes were also meaningful when compared with controls. In UV-C illumination, the before and after comparison exhibited statistically meaningful decreases in the white blood cell (WBC) count, hematocrit, and the platelet values. However, comparison with their corresponding controls revealed that changes in WBC and hematocrit were not statistically significant because similar trends were observed in the controls. With the violet light illumination, the before and after comparison showed a statistically significant decrease in platelet count. Nevertheless, the comparison with controls showed that this change was not significant given that the same trends were observed in the controls. Violet did not show any other significant difference from control, which indicates that its effects were minimal (for the entire analysis by CBC differential see Table S1).

In order to assess the potential toxicity of PDT, UV-C and violet light on peripheral blood mononuclear cells (PBMC) in whole blood, the level of DNA damage was determined, using the hOGG1-modified comet assay, before (0 h) and after exposure (5 h). Results from the experiments using the untreated blood samples in hOGG1- or T4endoV-modified comet assay did not show any significant changes at the 5 h timepoint, compared to control (Fig. 4A, B), during which the other samples were being treated with PDT, violet or UV-C. However, the results from the PDT experiment demonstrated a significant ($P < 0.0001$) increase in both 8-oxoGua and SB/ALS in blood samples after 5 h of exposure, compared to the level of DNA damage before PDT exposure (Fig. 4C). In contrast to PDT, UV-C did not induce SB/ALS after 5 h of exposure, compared to 0 h (Fig. 4D). However, as determined by T4endoV modified comet assay, we noted a significant ($P < 0.0001$) increase in the formation of CPDs, following 5 h of UV irradiation compared to the 0-h timepoint (an increase of 2.75% tail DNA). The results presented in Fig. 4E illustrate that the level of SB/ALS did not show any significant changes following 5 h of exposure with violet. Likewise, we saw no formation of CPD in PBMC after 5 h of violet exposure.

Discussion

Current state-of-art blood pathogen reduction technologies utilize photo-sensitizers or photoactivatable reagents (UV based) which are dependent on the light's penetration depth

through blood. Therefore, these technologies are used after light blocking RBCs have been removed. PDT, that is activated by light in ~ 700 nm - 1000 nm region, has been proposed for certain applications, because hemoglobin and water absorb less light in the region (23, 34, 35). However, pathogen inactivation, even in this optical window, can be partially hindered by RBC absorption (16). By reducing the thickness of the blood container for illumination, the penetration depth in various wavelengths becomes sufficient to inactivate the pathogens.

We developed a photosensitizer-antibody conjugate approach to target pathogens by using Chlorin E6 (a photosensitizer activated at 667 nm). By flowing the blood through a thin tube, we were able to inactivate high concentrations of pathogens in whole blood. PDT is based on the oxidative stress from locally induced, extremely short lived, reactive oxygen species (ROS) generated by photosensitization. The ROS are toxic to any cell in close proximity. CE6 was conjugated with antibodies to target *S. aureus* and MRSA in order to selectively target the pathogens while reducing non-specific damage to blood components.

The potential genotoxicity of PDT to nucleated blood cells was investigated using the Comet assay. A simplified method for the collection, storage, and comet assay analysis of DNA damage in whole blood was introduced by our group previously (33). Using this approach in the current study, the level of DNA damage in PBMC following PDT exposure was measured. To evaluate the effects of PDT exposure on induced levels of oxidatively generated damage to DNA nucleobases, the hOGG1 enzyme was used as a lesion-specific enzyme in the Comet assay. The differences between the effects in the absence and the presence of hOGG1 enzyme after 5 hs of PDT exposure showed a significant difference in the level of DNA damage indicating that PDT induced both SB/ALS and oxidized purines. This increased level of DNA damage following PDT exposure is similar to the finding of El-Hussein et al. (36) who showed that DNA strand breakage increased significantly in three cancer cell lines in response to PDT. Interestingly, a consistent high background level of SB/ALS and 8-oxoGua was detected in all unexposed and exposed samples before and after light exposure. We had previously shown the adverse effect of the temperature and duration of storage on the background level of DNA damage on PBMC (33). We speculate that the high background level of damage seen here may be due to the period of time for which these samples were frozen, combined with the effect of the two freezing/thawing cycles during the collection of the samples from the point of collection to the laboratory where the Comet assay was run. However, the induced level of damage after 5 h of exposure is still detectable, compared the level of damage before exposure.

PDT appears to induce several changes in the hematological profile according to the CBC differential results. Blood treated with PDT showed decreased hematocrit, increased MCHC and increased monocyte count compared to controls. The decrease in hematocrit and increase in MCHC are attributed to minor reductions in the RBC count while hemoglobin levels stay constant. This indicates that RBC were slightly stressed by the process and perhaps there was minor hemolysis. However, these changes are within the reference range and thus could be considered minor effects. The elevation in the monocyte count is difficult to explain, because, while there is no source of monocytes (usually produced in the bone marrow). The light intensity of the 660 nm LED source was measured at 0.355 μ W and the

light dose was calculated to be $\sim 0.0047 \text{ J/cm}^2$. The light dose delivered is lower than this value due to the minor turbidity of PDMS tube. The light dose was calculated assuming that the light came from one direction and the tubes were illuminated for 3 h constantly. While the illumination occurred inside a mirror box, it is difficult to estimate the light intensity from all directions. Also, we need to consider that the blood is in constant flow therefore the duration of the illumination is significantly shorter than 3 h. A typical PDT light dose in clinical settings is in the tens to hundreds of J/cm^2 , which is higher than the values used in this work. The observed change in blood composition might be attributed to the high photosensitizer concentration. It should be noted that the toxicity study was performed *in-vitro* without bacteria, thus our study overestimated the toxic effect (given that none of the conjugates were bound to bacteria).

There are several aspects of the PDT based approach that can be improved. First, this approach is useful only when the contaminant is known. Otherwise, the CE6-Ab conjugate would be non-specifically toxic to nearby cells. Second, this approach requires fine tuning of dosage by considering parameters such as photosensitizer concentration, light intensity and duration. Without fine tuning, low doses may not reduce pathogens to acceptable levels, while high doses may cause collateral damage to normal blood cells. Utilization of a photosensitizer with affinity towards nucleic acids offers a solution. But, the potential of non-specific damage remains, unless the unbound photosensitizers are removed. This is a common problem in pathogen reduction technologies that rely on DNA binding reagents.

The UV-C and violet approaches described in this report do not require addition of photoactivatable reagents. Current PRTs utilize UV-A waveband to activate reagents that are only effective in the absence of RBC (10, 12, 13). Our results demonstrate that PRTs can be achieved directly with whole blood without using additional photoactivatable reagents (10, 12, 13) by applying our approach of flowing blood through a thin tube. The germicidal effects of UV are well known (17, 18) and violet light was used for treatment of bacterial infections (28, 29).

The health risks of UV irradiation, include DNA damage, mutagenesis, and carcinogenesis, and are well documented (37, 38). However, in our toxicity investigations of UV-C and violet, we observed only minor effects on genotoxicity to WBCs, and hematological profile. As mentioned earlier, the incorporation of lesion-specific repair enzymes such as T4endoV in the Comet assay brings greater information on the types of lesions present in the DNA. The data gathered here shows no difference in the mean basal levels of damage in the absence of T4endoV between the blood samples prior and 5 h post irradiation, indicating that the dose used in this study does not induce SB/ALS in the DNA. However, the UV-C dose used in this study induced a significant level of CPDs post exposure, compared to the level of CPDs before UV-irradiation. Although the amount of UV-induced CPDs was significantly different after 5 h of irradiation, it only represents 2.74% of tail DNA which may be considered as a very low level of induced-CPDs, which is very likely to be repaired by the DNA repair systems in the cell.

In CBC differential results, increases in MCHC were observed with UV-C, which may indicate slight hemolysis. However, the changes were less than that observed with PDT and

they were within the normal reference range (39). The total UV-C exposure dose was calculated to $\sim 0.028 \text{ J/cm}^2$ (accounting for the reduced transmittance of UV-C light through PDMS tube $\sim 70\%$, refer to Section 2.4 for transmittance measurement), but distributed partially across the blood sample due to recirculation. The dose used is similar to literature reported values (40). Violet light did not exhibit any statistically significant difference from controls in both genotoxicity and CBC differential results. The light dose for the violet light was 2.4 J/cm^2 . This value is significantly higher than that of UV-C and indicates that the germicidal capability of violet light is weaker. The minimal damage observed in WBC with UV-C and violet may be because the blocking some of the light by the RBCs could protect WBCs while exerting sufficient killing of bacteria. WBCs are also equipped with more robust repair mechanisms, compared to smaller cells and pathogens with limited repair capability.

We believe that bacterial inactivation in the proposed flow system occurs at close proximity to the tube walls. Thus, some pathogens may evade exposure to light. Overall, the highest pathogen reduction was achieved with the combinations of PDT + UV-C + CAP in *S. aureus* and UV-C + CAP in MRSA (the triple combination was not pursued with MRSA, because we had $\sim 98\%$ reduction by combining two techniques). UV-C illumination is the most promising and straightforward technique for pathogen inactivation among the three light illumination methods studied, primarily because it does not require additional reagents and is effective in inactivating pathogens. In combination with immunocapture it improves pathogen reduction, without adding toxicity concerns. The addition of immunocapture is unique and offers a solution to improve PRT in whole blood. However, immunocapture also requires identification of pathogen to be effective. Recent efforts by other groups have developed human opsonins that capture a wide array of pathogens and toxins (41). These types of molecules can be utilized for immunocapture as well as for photosensitizer conjugation.

In conclusion, we have presented three promising new approaches that enable pathogen inactivation directly in whole blood, by employing inexpensive thin tubing. These can be adapted to current PRTs for blood transfusion and for manufacturing of blood products. Further studies are required to increase the efficiency of pathogen reduction to acceptable levels.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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conducted all the Comet assay experiments and wrote the relevant sections. A.G. and G.K. wrote the manuscript. All authors edited, reviewed and approved the final draft of the manuscript.

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Figure 1.
 (a) Diagram of the flow system. (b) The principle of pathogen inactivation in whole blood enabled by recirculation through thin channel.

Figure 2.

Experimental set-up for three light-illumination techniques. (a) The PDT technique utilized a 660 nm LED array located on top of the illumination chamber (mirror box). Blood was circulated through the tube using a peristaltic pump, part of the tube was inserted in the chamber for illumination. (b) The UV-C technique used UV-C lamp installed inside the illumination chamber and, as before, part of the tube was inserted in the chamber and a pump was used for blood circulation. (c) For the violet technique, two violet LEDs were used. The coiled tube was placed between the two LEDs facing each other. A fan was used to remove excess heat.

Figure 3. Summary of pathogen reduction of each technique for (a) *S. aureus*; and (b) MRSA. (n=5). The values represent the percentage of remaining bacteria in the reservoir compared to control [100 x (control – exposed blood) / control]. Abbreviations: PDT: photodynamic therapy treated, UV-C: UV-C illuminated, violet: violet illuminated, PDT + CAP: Combination between PDT and immunocapture, UV-C + CAP: combination between UV-C and immunocapture, violet + CAP: combination between violet and immunocapture, PDT + UV-C + CAP: combination among PDT, UV-C, and immunocapture.

Figure 4.

Level of (A) SB/ALS and 8-oxoGua, or (B) SB, ALS and CPDs in whole blood samples collected 0 h or 5 h following no exposure, (C) PDT exposure, (D) violet irradiation or (E) violet exposure using hOGG1-modified or T4 endoV-modified alkaline comet assay. DNA damage was expressed as % tail DNA. Each data point represents the mean of 600 (A, B, C, D) determinations from three different blood samples or 400 (E) determinations from two different blood samples, all from two individual experiments, $\pm$ SEM (ns= non-significant, *** $P < 0.001$).