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RESEARCH ARTICLE

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Direct inactivation of SARS-CoV-2 by low level blue photobiomodulation LED at 470, 454 and 450 nm

Luisa Zupin[1]* | Rossella Gratton[1] | Margherita Milani[2] | Libera Clemente[3] | Francesco Fontana[3] | Maurizio Ruscio[3] | Sergio Crovella[4]

[1]Institute for Maternal and Child Health—IRCCS "Burlo Garofolo", Trieste, Italy

[2]Department of Medical Surgical and Health Sciences, University of Trieste, Trieste, Italy

[3]Division of Laboratory Medicine, University Hospital Giuliano Isontina (ASU GI), Trieste, Italy

[4]Biological Science Program, Department of Biological and Environmental Sciences, College of Arts and Sciences, University of Qatar, Doha, Qatar

***Correspondence**
Luisa Zupin, Institute for Maternal and Child Health, IRCCS Burlo Garofolo, 34137, Trieste, Italy.
Email: luisa.zupin@burlo.trieste.it

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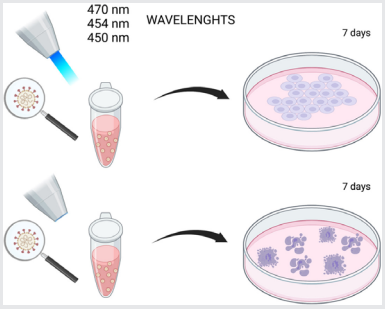
Abstract



Blue light has been already reported as able to counteract different types of microorganisms including Gram-positive and Gram-negative bacteria, fungi and viruses, especially the enveloped ones. It has been reported that both blue and visible light can efficiently impact SARS-CoV-2 by affecting its ability to replicate in in vitro cellular models of infection. In this study, blue light at 450, 454 and 470 nm was tested on SARS-CoV-2 to evaluate the residual viral infectious potential on Vero E6, Caco-2 and Calu-3 cells, after the irradiation of viral particles. Following 12′ of irradiation at 40 mW/cm^2, a drastic block of viral amplification was observed. Indeed, at 7 days post-irradiation/infection the viral load was the same as the one measured 1 day post-irradiation/infection, and cellular viability was maintained showing similar levels to the noninfected control cells. Taken together our results indicate that blue LED lamps can be considered as a cheap and convenient tool for SARS-CoV-2 disinfection.

KEYWORDS

antiviral, blue LED, disinfection, photobiomodulation therapy, SARS-CoV-2

1 | INTRODUCTION

Blue light is known to exert a wide spectrum of antimicrobial properties against different types of pathogens including both Gram-negative and Gram-positive bacterial strains, fungi and viruses [1–4].

Endogenous photosentizers present in bacteria and fungi, such as porphyrins, are photoexcited by blue wavelengths and induce the production of high levels of cytotoxic reactive oxygen species ultimately leading to cell death [3, 4]. On the other hand, little is known about the antiviral properties of blue light, and the mechanisms underlying these effects should be further investigated.

Recently, we reported the antiviral effect of laser light at 445 nm against Herpes simplex virus type-1 (HSV-1) [1] and Zika virus (ZIKV) in in vitro cellular models of infection [2]. Our results indicate that the treatment was able to induce a decrement in the viral load especially when the virus was irradiated alone and then transferred to the cell cultures, hence possibly suggesting that the light exerts a direct effect on viral structure.

In December 2020, we observed the effect of three different wavelengths (450, 454 and 470 nm) against the viral agent of the current pandemic, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [5]. Specifically, by using light emitting diode (LED) devices,

we detected an efficient abatement of the viral load at 24 and 48 h in cells that were infected for 1 h and then irradiated. We hypothesized that SARS-CoV-2 might result in being more susceptible to the irradiation when it possesses an intracellular localization, probably due to the interference caused by blue light with the viral replication machinery [5].

In accordance with our previous results, a study by de Santis et al. [6] displayed the reduction of the viral load when SARS-CoV-2 was first illuminated for 15, 30, 45 or 60 min by visible LED light (peak at 413 nm) and then inoculated to a cell culture. In a recent article by Stasko et al., the 425 nm LED light was also efficient against SARS-CoV-2, SARS-CoV-1 and MERS-CoV in Vero E6 cells and primary in vitro human 3D tracheal/bronchial tissues [7].

The coronavirus disease 2019 (COVID-19) is efficiently limited by the ongoing vaccination campaign endorsed by public health policies; nevertheless, the spread of SARS-CoV-2 variants raise questions regarding the coverage efficacy of the available vaccines. Moreover, the high mutation rate of this pathogen drives its rapid evolution, hence potentially impacting its virulence and transmission capacity [8].

The urgent need to assess strategies able to restrain these issues is highly emboldened, and in this critical context blue light might represent a promising and intriguing approach to successfully counteract viral resistance, acting as a potent disinfectant tool.

Taking into account our previous data [5], in this study the disinfection potential of blue light at 450, 454 and 470 nm was tested on the virus alone, by initially irradiating SARS-CoV-2 in a liquid solution and then by evaluating its residual infectivity on three different cell lines, namely Vero E6, Caco-2 and Calu-3. In our previous study, an effective outcome was registered uniquely following the irradiation of cells that were pre-infected with SARS-CoV-2 for 1 h, while in our novel experimental setting we tested a higher fluence (30 J/cm^2), which showed low cytotoxicity levels, to determine whether blue light might be employed as a tool for SARS-CoV-2 disinfection and decontamination procedures.

2 | EXPERIMENTAL SECTION

2.1 | Cell line and LED irradiation

Vero E6 normal renal epithelial cell line from *Cercopithecus aethiops* (ATCC CRL-1586), Caco-2 human colorectal adenocarcinoma epithelial cell line (ATCC HTB-37) and Calu-3 human lung adenocarcinoma epithelial cell line (ATCC HTB-55) were employed. Vero E6 were maintained in MEM + 10% fetal bovine serum (FBS), 2 mM glutamine and 100 U/ml penicillin/streptomycin (Euroclone, Pero, Italy). Caco-2 were cultured in Minimum Essential Medium Eagle (MEME, M5650, Merck KGaA, Darmstadt, Germany) + 20% fetal bovine serum, 2 mM glutamine and 100 U/ml penicillin/streptomycin (Euroclone). Calu-3 were cultivated in 1:1 Dulbecco's Modified Eagle Medium (DMEM): Ham's F12 + 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin/streptomycin, 1% nonessential amino acids (Euroclone) and 1 mM sodium pyruvate (Merck KGaA). For the infection assays, 10^5 cells were seeded in 24 multiwells plates. For viral irradiation and absorption procedures, DMEM without phenol red (BE12-917F, Lonza, Basel, Switzerland) + 2% FBS, 2 mM glutamine and 100 U/ml penicillin/streptomycin (Euroclone, Pero, Italy) was used. Then, for the maintenance of cell cultures during the entire experimental period of 7 days, the medium was replaced with the same mediums that were above described as specific for each cell line, yet supplemented with only 2% of FBS.

2.2 | LED irradiation

Three LED devices at 450, 454 and 470 nm (BLUù series, developed by UV-Core based on Cortem Group's EVML lighting Fixture, Cortem S.p.A, Villesse, Italy) were tested (irradiance of 40 mW/cm^2, fluence 30 J/cm^2, continuous waves) in dark conditions.

The temperature was measured, at the site of irradiation at the beginning and at the end of the protocol by using a thermo scanner (Mestek, Westfield, MA). The measurement was conducted under the hood at room temperature, immediately after the removal of the plate from the 37°C incubator, then after irradiation the temperature was registered again.

To test the cytotoxicity of blue LED, 10^4 cells were seeded in 96 multiwell plates and irradiated in DMEM without phenol red (BE12-917F, Lonza, Basel, Switzerland) + 2% FBS, 2 mM glutamine and 100 U/ml penicillin/streptomycin (Euroclone). Following the treatment, cells were cultured in their medium +2% FBS. After 1 day or 7 days post-irradiation/infection, crystal violet staining was employed to assess cellular viability. Briefly, the medium was removed from each well and replaced with crystal violet (10% in phosphate buffer saline—PBS) solution. After 30 min, wells were washed three times with water and air dried. Next, 200 µl of lysis solution (1% Dodecyl sodium sulfate in PBS) was added to the wells. Next, following 30 min the lysis solutions were diluted (1:4) in water and the absorbance of 200 µl deriving from each well were read in a 96 multi-wells plate using a spectrophotometer set at 600 nm.

2.3 | LED antiviral activity

SARS-CoV-2, kindly provided by the BLS3 facility of San Polo Monfalcone hospital (GO, Italy) and previously employed in other experiments [5, 9] was used at a multiplicity of infection (MOI) of 0.02.

To evaluate the possible direct effect of the blue light on the virions, SARS-CoV-2 was irradiated alone in a final volume of 100 μl, and then transferred to the three cell lines for 1 h to allow viral adsorption. At the end of the incubation period, supernatants were removed, samples were washed in PBS, and fresh medium was added.

Analyses were conducted at two different time points: 1 day post-irradiation/infection, to test the early effects of blue light on viral particles; 7 days post-irradiation/infection, to determine the possible residual infectivity that might be able to establish a long-term infection.

To quantify the viral load, 15 μl of cell culture supernatants were collected and thermolyzed with 45 μl of water (98 °C for 3 min, by 4 °C for 5 min). The viral RNA was then measured by using real time PCR with CDC primers and a fluorescent probe (Eurofins, Luxembourg) specific for the viral gene *N*, and by employing a standard derived from nCoV-CDC-Control Plasmid (Eurofins), as previously described [5, 9].

At the end of the experimental procedure, cellular viability was determined using crystal violet staining as previously described (Section 2.2).

The effects of irradiation on the integrity of virions was assessed with the RNase protection assay. Briefly, following illumination, 15 μl of samples and controls were hydrolyzed by the RNase enzyme (1 μg of Ribonuclease A R4875, Merck KGaA) for 30 min at 37°C. Next, 45 μl of water were added to the mix and samples were submitted to thermolysis (98°C for 3′, 4°C for 5′) and quantified as above described.

2.4 | Statistical analysis

The Kruskal–Wallis (KW) non-parametric test corrected for multiple comparisons with Dunn's test was employed to compare cells treated with the non-irradiated virus with cells that were infected with the irradiated SARS-CoV-2 using R statistical software [10]. Each experimental setting was performed in eight replicates in two independent tests.

3 | RESULTS

Despite at 1 day post-irradiation/infection we did not highlight differences in the viral load between cells inoculated with the non-irradiated virus if compared with those infected with the irradiated viral particles, we registered a significant change at 7 days post-irradiation/infection in all three cell lines.

At 7 days post-irradiation/infection, in Vero E6 cells the viral load of the control cells (infected with non-irradiated virus) reached 10^9 RNA viral copies/ml, while the viability decreased at 50% if compared with non-infected (NT) cells. Instead, while considering cells inoculated with the irradiated virus, the viral load stabilized at 10^6 RNA viral copies/ml, same amount that was detected at 1 day post-irradiation/infection, probably due to the presence of noninfectious virions or RNA remnants that were not completely removed by the washing step with PBS (KW test LED 450 nm vs. not irradiated virus p value = 0.001, KW test; LED 454 nm vs. not irradiated virus p value = 0.001; KW test LED 470 nm vs. not irradiated virus p value = 0.02; Figure 1A), while the survival rate was comparable to non-infected cells (KW test LED 450 nm vs. not irradiated virus p value = 0.00001; KW test LED 454 nm vs. not irradiated virus p value = 0.002; KW test LED 470 nm vs. not irradiated virus p value = 0.000004; Figure 1B).

In Caco-2 cells similar results were observed. Specifically, at 7 days post-irradiation/infection the replication of the non-irradiated virus reached 10^8 RNA viral copies/ml, meanwhile the irradiated one stabilized at 10^5 RNA viral copies/ml (KW test LED 450 nm vs. not irradiated virus p value = 0.0002, KW test; LED 454 nm vs. not irradiated virus p value = 0.0005; KW test LED 470 nm vs. not irradiated virus p value = 0.003; Figure 1C). In Caco-2 cells, no significant changes in cellular viability were registered after 7 days post-irradiation/infection when comparing noninfected cells, cells infected with non-irradiated virus and cells infected with irradiated viral particles (Figure 1D).

Also, in Calu-3 cells blue light seemed to block viral replication. At 7 days post-irradiation/infection, the viral load registered in control cells infected with non-irradiated virus reached 10^8 RNA viral copies/ml versus 10^6 RNA viral copies/ml in presence of the irradiated virus (KW test LED 450 nm vs. not irradiated virus p value = 0.01, KW test; LED 454 nm vs. not irradiated virus p value = 0.01; KW test LED 470 nm vs. not irradiated virus p value = 0.001; Figure 1E). In Calu-3, an increment in cellular viability was detected in the cells infected with the irradiated viral particles when compared with those treated with non-irradiated SARS-CoV-2 (85–100% vs. 50%; KW test LED 450 nm vs. not irradiated virus p value = 0.01, KW test; LED 454 nm vs. not irradiated virus p value = 0.001; KW test LED 470 nm vs. not irradiated virus p value = 0.05; Figure 1F).

A decrement of the viral load was achieved, using the RNase protection assay following the irradiation

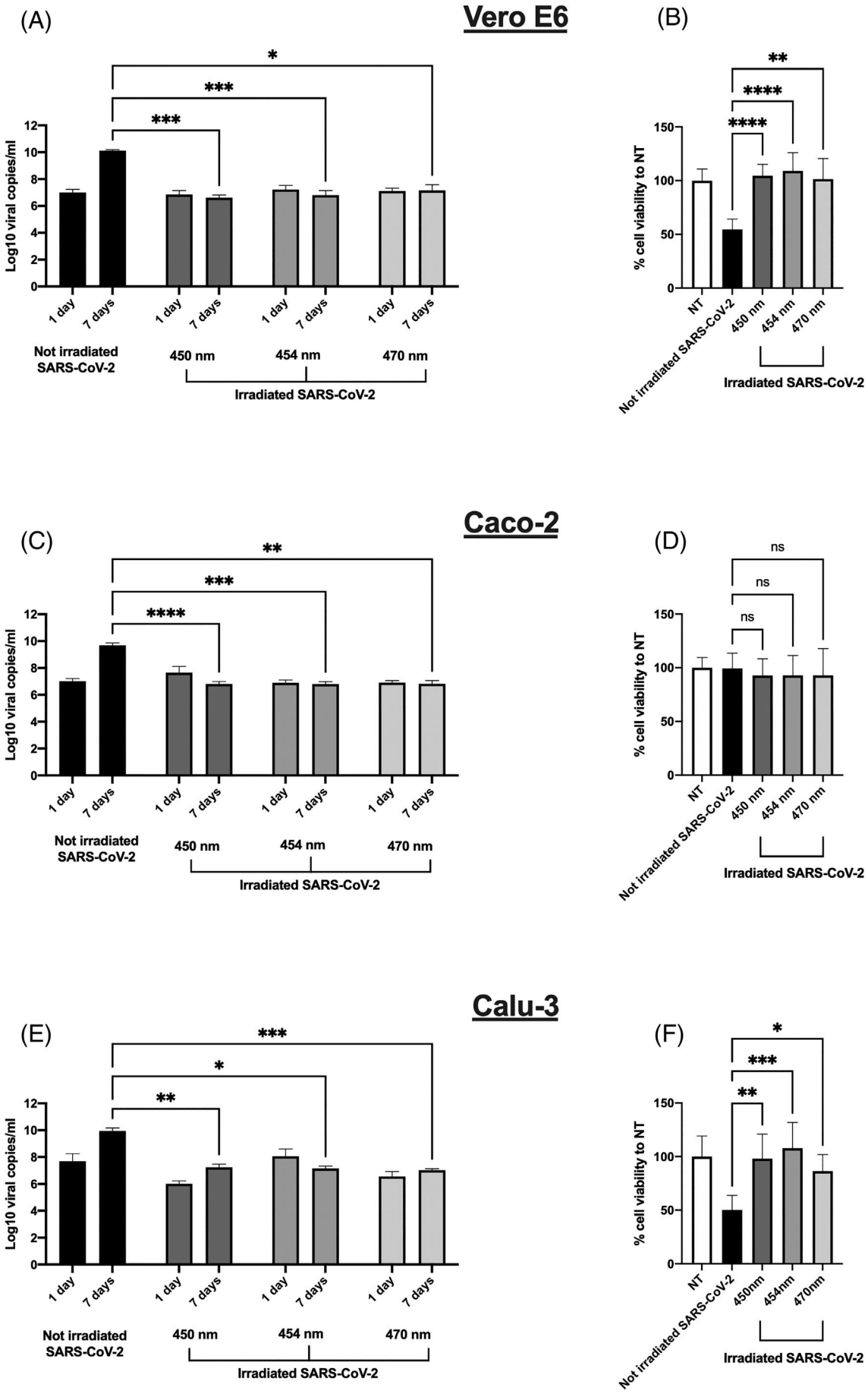


FIGURE 1 Legend on next page.

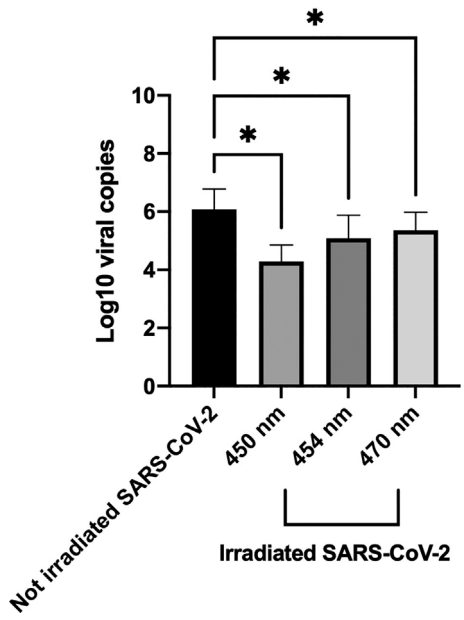


FIGURE 2 Effect of blue LED light at 450, 454 and 470 nm on virion integrity assessed by RNase protection assay. The viral load was displayed as Log10 viral copies/ml. Kruskal–Wallis test corrected for multiple comparison with Dunn's test was employed for the comparison between irradiated and nonirradiated virus (*$p < 0.05$, **$p < 0.01$)

protocols for all three tested wavelengths (KW test LED 450 nm vs. not irradiated virus p value = 0.03; KW test LED 454 nm vs. not irradiated virus p value = 0.03; KW test LED 470 nm vs. not irradiated virus p value = 0.01; Figure 2).

The protocols showed modest cytotoxic effect for all of the three tested cell lines (Table 1), since cellular viability reached about 80% after irradiation if compared with the non-irradiated control cells.

The possible thermal effect was monitored and an increment of 10°C was registered at the end of the treatment (Table 2). Nonetheless, it is relevant to consider that temperature variations fall into in a physiological range (~37°C), thus allowing us to exclude a thermal antiviral effect, and therefore strongly confirming the effective photochemical action of blue lights.

4 | DISCUSSION

The purpose of this study was to continue the investigation on the potential effects of blue light in counteracting SARS-CoV-2. Based on our previous results obtained with LED lamps at 450, 454 and 470 nm [5], we employed a higher fluence (30 J/cm^2) that was tested on the virus alone prior to the establishment of a cellular infection. Three different permissive cell lines were employed and include the primate non-human derived Vero E6 cells, and the human Caco-2 and Calu-3 adenocarcinoma epithelial cell lines, the latter recapitulate suitable models for colon and lung cancer tissues.

Our results were almost comparable between the three different cell lines. When comparing the viral load between the cells inoculated with not irradiated and irradiated virus a ~ 3 Logs (~1000 times) of reduction were detected in Vero E6 and Caco-2 cells, and ~2 Logs (~100 times) of reduction in Calu-3 cells. But the interesting finding is related to the observations that non-irradiated virus replicated and amplified in all tested cell cultures, while in the case of irradiated SARS-CoV-2 the RNA viral load stabilized at the same level of that registered at 1 day post-irradiation/infection indicating a non-competent replicative virus after irradiation. Therefore, blue light was able to block the virus that did not generate an established infection at the end of the experimental period set at 7 days post-irradiation/infection.

The residual viral RNA detected at day 7 could be probably due either to the washing steps that did not eliminate completely the virus, or to the attachment of non-replication competent viral particles to the cellular membranes.

Being aware that the selected protocols were slightly cytotoxic (about 80% of viable cells at 1 day and 7 days post-irradiation/infection if compared with the non-irradiated

FIGURE 1 Effect of blue LED light at 450, 454 and 470 nm on SARS-CoV-2. The virus was irradiated in a multiwell plate and then transferred to a monolayer of cells (Vero E6, Caco-2 and Calu-3) for 1 h. The RNA viral load was tested at 1 day and 7 days post-irradiation/infection. Non-irradiated virus and viral particles irradiated with LED at 450, 454 and 470 nm are displayed. The viral load was quantified from the supernatants and showed as Log_{10} viral copies/ml. At the 7 days post-irradiation/infection, the viability of the cells non-infected (NT) and treated with irradiated and non-irradiated virus was evaluated. Results are reported as the percentage of viable cells normalized to NT cells. (A) SARS-CoV-2 RNA viral load in Vero E6 cells at 1 day and 7 days post-irradiation/infection. (B) Cell viability in Vero E6 cells at 7 days post-irradiation/infection. (C) SARS-CoV-2 RNA viral load in Caco-2 cells at 1 day and 7 days post-irradiation/infection. (D) Cell viability in Caco-2 cells at 7 days post-irradiation/infection. (E) SARS-CoV-2 RNA viral load in Calu-3 cells at 1 day and 7 days post-irradiation/infection. (F) Cell viability in Calu-3 cells at 7 days post-irradiation/infection. Kruskal–Wallis test corrected for multiple comparison with Dunn's test was employed for the comparison between the tested groups (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

TABLE 1 Viability of the three cell lines (Vero E6, Caco-2 and Calu-3) at 1 day and 7 days post-irradiation/infection

	1 day				7 days			
	450 nm	454 nm	470 nm	NT	450 nm	454 nm	470 nm	NT
Vero E6	97%	99%	99%	100%	80%	76%	87%	100%
Caco-2	92%	91%	81%	100%	104%	74%	68%	100%
Calu-3	86%	98%	87%	100%	80%	88%	85%	100%

Note: The results are presented as percentages compared with non-infected (NT) cells (set at 100% of viability).

TABLE 2 Temperature monitoring prior and after irradiation procedures

Wavelength	Before irradiation	After irradiation
470 nm	24.6 °C	36.0 °C
454 nm	23.6 °C	37.2 °C
450 nm	25.0 °C	37.0 °C

ones), we were not able to irradiate cells directly with these LED parameters.

Nevertheless, this aspect did not affect the impact of our study since our aim was to assess the potential activity of blue LED as a disinfectant tool able to penetrate into a liquid medium. To note, in the context of disinfection and decontamination classical ultraviolet lamps are generally regarded as a highly efficient and well-established tool. Nevertheless, these devices possess some nonnegligible limitations such as the inability to penetrate deeply into fluids, the induction of polymer degradation, and the emergence of phototoxic responses in humans [11]. The setting of our experimental model allowed us to overcome the previously described limitations by employing blue LED light.

Our data can be considered in agreement with those reported in the study conducted by de Santis et al. [6] although with important differences in the experimental setups: their irradiation is quite prolonged (15, 30, 45 and 60 min) while our protocol lasted 12 min; they used visible light with a range of wavelengths from 400 to 780 nm, while we used a specific set of wavelengths (450, 454 and 470 nm).

Another study recently conducted by Rathnasinghe [11] explored the impact of 405 nm light on SARS-CoV-2, obtaining viral inactivation with an irradiance ranging from 0.035 to 0.6 mW/cm^2. The authors irradiated the virus for 24 h and observed a reduction after at least 4 h of illumination. Although the irradiance was lower than the one tested in our study, the fluence and energy delivered were higher and not comparable to our experimental setting.

Very recently, Staskos et al. [7], employed LED light with a peak at 425 nm on SARS-CoV-2, SARS-CoV-1 and MERS-CoV. Irradiating already infected cells, an inhibition of virus replication was observed at 24 and 48 h in Vero cells with dosages ranging from 7.5 to 60 J/cm^2 (50 mW/cm^2), and in a similar way blue LED hindered SARS-CoV-2, when it was irradiated alone and then transferred to cell culture. LED at 425 nm also resulted to be effective in an infected 3D human tracheal/bronchial-derived epithelial tissue following irradiation twice a day for 4 days. Although the doses were similar to those employed in our study, in our experiments at 24 h no differences in viral load values were observed, while a dramatic effect was achieved at 7 days post-irradiation/infection.

Our findings were in accordance with our previous results reporting that 445 nm laser light was effective especially when HSV-1 and ZIKV were irradiated alone [1, 2].

Other two studies investigated the effect of 405 nm [12] and 455 nm [10] wavelengths on Bacteriophage phi6 showing a reduction of the load, with log reduction doses of 430 and 2130 J/cm^2, respectively. It has been proposed [10, 12] that the mechanism responsible for viral inactivation resides in the incorporation of photosensitizers during the assembly of the envelope inside host cells. Indeed, the cellular membrane is associated with molecules able to respond to blue light including porphyrins, flavins and NADH [13].

In the current study, with the RNase protection assay, a decrement in the viral load was achieved after irradiation. This assay, based on the principle that the RNase enzyme degrades the free RNA that is not protected by the virion's envelope, strongly indicates that blue light might partially disrupt the virus's integrity in accordance with the hypothesis assuming the presence of photosensitizers within the envelope. Since the effect was not complete (a decrement of 1 Log was observed), we may hypothesize that the antiviral activity of blue light might act through different mechanisms including for instance the induction of the impairment of the virion's integrity, or the blocking of some vital functions of SARS-CoV-2. Indeed, it has been reported that visible light inactivated murine leukemia virus due to a polymerase processivity

defect [14], meanwhile ultra-pulsed visible laser caused aggregation of capsid and tegument protein of murine cytomegalovirus [15]. Moreover, in the study by Stasko et al. [7] a greater reduction in infectious titer was observed by using the $TCID_{50}$ test respect to the RT-qPCR detection, possibly suggesting the RNA nuclei acid is only one of the targets of antiviral action.

The monitoring of temperature during irradiation showed that from the initial 25°C (room temperature), an increment up to 37°C was registered. The first measurement was conducted immediately after the withdrawal of the plate from the 37°C incubator, obviously, the plate quickly dissipates the warmth when deposited on the hood at room temperature (25°C). Then the plates were irradiated for 12′ at the end of the treatment the temperature was registered (37°C). Therefore, the irradiation and direct photo-virucidal effect of blue LED really occurs at physiological temperature, since at the end of the irradiation, the medium containing the virus was at 37°C. Therefore, a thermal distrusting effect was avoided confirming the photoinactivation due to blue light.

Very recently, the usage of blue light was proposed as a tool for endotracheal tube disinfection in intensive care units [16]. The authors successfully tested the efficacy of blue light on a strain of *Staphylococcus*, proposing this technology as an alternative to avoid ventilator associated pneumonia. However, our results indicate that this procedure could be advantageous also for the inactivation of SARS-CoV-2 or other viruses. Thus, the risk of common nosocomial infections together with SARS-CoV-2 contamination associated with endotracheal tubes could be reduced.

5 | CONCLUSIONS

Our findings suggest the potential employment of blue light as a successful disinfection treatment. With a very short time of irradiation (12 min) with respect to those reported in literature, we were able to block the establishment of a viral infection in vitro until 7 days post-irradiation/infection.

LED blue light is a cheaper alternative of blue laser and could be readily available from commercial sources. These results open new potential effective applications of blue LED for different purposes such as the inactivation of pathogens present in biological liquids. Moreover, by means of a single technology, a potent decontamination of virus but also of bacteria and fungi [4] could be achieved.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Luisa Zupin, Rossella Gratton, Margherita Milani, and Libera Clemente were involved in performing the experiments and writing—original draft. Francesco Fontana and Maurizio Ruscio were involved in supervision of the experiments conducted in the BLS3 facility, writing—review and editing. Sergio Crovella was involved in conceptualization of the study, writing—review and editing, project management.

DATA AVAILABILITY STATEMENT

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

ORCID

Luisa Zupin https://orcid.org/0000-0001-5886-9129

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