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ORIGINAL PAPERS

The virucidal potential effects of violet-blue light on influenza D virus

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Abstract

Influenza D virus (IDV) is a novel influenza virus, first isolated from swine with influenza-like symptoms in the USA in 2011. To date, IDV circulation has been reported in various animal species such as cattle, pigs, horses with the ability to expand its range of hosts. UV radiation has been widely used for the disinfection of various sources such as water, air, and surfaces, especially in places at greater risk of contamination by viruses and bacteria, such as hospitals and health facilities. The aim of this study was to evaluate the potential virucidal effect of a violet-blue light against IDV. Viral suspension of IDV was exposed to a violet-blue light (405 nm) for different times (radiant exposures): 22 min and 30 s (5.4 J/cm^2), 45 min (10.8 J/cm^2), 90 min (21.6 J/cm^2), 180 min (43.2 J/cm^2), and 360 min (86.4 J/cm^2), and different temperatures (room temperature, 4 and 37 °C). At the end of exposure, virus titration was performed on MDCK cells. After violet-blue light exposure, a viral titre reduction proportional to exposure time was observed: 0.228 $\log_{10}$ after 22 min and 30 s, 0.668 $\log_{10}$ after 45 min, 0.940 $\log_{10}$ after 90 min, 1.375 $\log_{10}$ after 180 min and 2.293 $\log_{10}$ after 360 min. Differences were observed among temperatures of exposure, with the greatest virucidal effect observed at room temperature. As reported for other respiratory viruses, this violet-blue light can potentially be used to reduce IDV spread in potentially hotspot areas for animals and humans.

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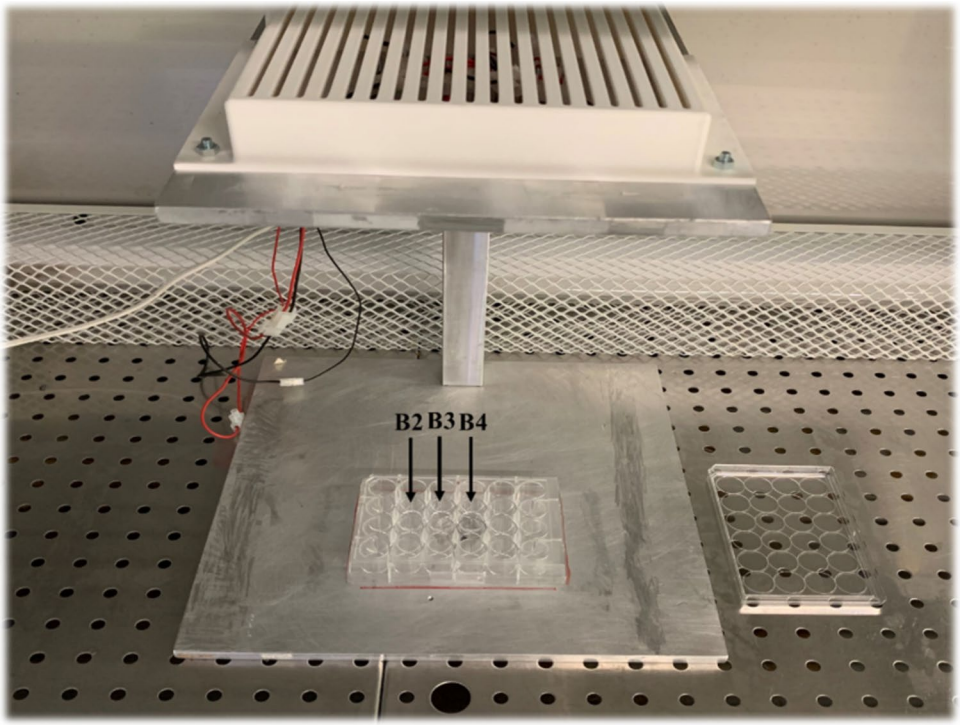
Graphical Abstract



Keywords Influenza D virus · Violet-blue light · Viral inactivation · Disinfection

1 Introduction

Influenza D virus (IDV) belongs to the Orthomixoviridae family, which includes three other species of influenza viruses: influenza A virus (IAV), influenza B virus (IBV) and influenza C virus (ICV). In 2016 IDV was officially classified as a new species belonging to the genus *Deltainfluenzavirus* [1].

IDV, like ICV, has a single-stranded, segmented RNA genome composed by seven segments that express nine different proteins [2]. Segment 4 encodes the hemagglutinin-esterase fusion (HEF) protein, the only surface glycoprotein, that acts as hemagglutinin (HA) and neuraminidase (NA) of IAV and IBV, serving for viral receptor recognition and binding the host cell [3].

IDV was first isolated in April 2011 in Oklahoma from swine showing influenza-like symptoms [4], and then it has been reported in several animal species like cattle, ovine, horses, dogs and other ruminants (camels, goats, and sheep) [2, 5, 6]. Many epidemiological studies revealed IDV isolation in cattle from many geographical areas, and a higher IDV prevalence in cattle compared to other species [3, 7], suggesting cattle as primary reservoir. Further studies have reported the presence of IDV antibodies in humans [8], especially in those who are in close contact with animals [9–11], suggesting that this type of virus can expand its host range.

The feature that makes IDV different from other influenza viruses is its stability in case of temperature and acidity changes [12]. For example, IAV can be inactivated by various reagents like sodium hypochlorite, ethanol, aldehydes, and common household agents, while IDV showed to be insensitive to these agents due to its strength characteristics [13]. IDV replicates well in cell cultures at 37 °C, temperature prevailing in the lung [14] and it is infectious even when exposed to a temperature of 53 °C for 2 h, retaining about 40% of its viral titre [15]. Similarly, IDV preserves its infectivity, about 80% of its infectious titre, when exposed to an acidic environment (i.e. pH 3.0 for 30 min), while IAV, IBV and ICV are completely inactivated under this condition [15].

Ultraviolet (UV) radiation has been widely used for the disinfection of various sources such as water, air, and surfaces [16], especially in places at greater risk of contamination by viruses and bacteria, such as hospitals and health facilities [17]. Specifically, UVC light is the most germicidal wavelengths (220–280 nm) of UV radiation [18, 19]. However, other frequencies in or near the UV

spectrum are also widely used to support standard disinfection procedure.

Recently, the antimicrobial properties of violet-blue visible light have emerged as an area of increasing research, especially the wavelengths in the region of 405 nm that have proved effective for the inactivation of microbial species [20–23]. Studies have demonstrated a greater susceptibility of microbial cells than mammalian cells, potentially providing the ability to inactivate microbes in contaminated tissues while sparing eukaryotic cells [24, 25]. Therefore, it could be used to control microbial populations in various environments [21]. This aspect is critical because contamination occurs in the presence of people, and the ability to take advantage of continuous decontamination would reduce the risk of infection in healthcare and non-healthcare settings [26–29]. The mechanism of action of 405 nm violet-blue light is a photodynamic inactivation [30, 31]. The exposure to light of this wavelength induces an oxygen-dependent photo-excitation reaction within exposed microorganisms, where excited porphyrins react with oxygen or cell components to produce reactive oxygen species (ROS). ROS, including singlet oxygen ($1O_2$), superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl groups (OH), are chemically reactive free radicals that play a crucial role in cell signalling and homeostasis. Still, their overproduction becomes toxic to cells by altering the redox balance [32]. This causes significant damage to cellular structures through the oxidation of cellular macromolecules such as proteins, lipids, nucleic acids, NADH/NADPH, and soluble thiols [26]. Because microbial and mammalian cells contain intracellular porphyrins, these porphyrins can be photosensitized during exposure to violet-blue light, resulting in the overproduction of ROS. It has been observed that cyclobutane-pyrimidine dimers (CPDs) are primarily responsible for direct DNA damage among all photo products, such as single-strand breaks and oxidation of purines and pyrimidines [33].

Several studies have shown that 405 nm light has a broad spectrum of activity, including antibiotic-resistant bacteria such as MRSA or HCA-associated organisms, including *Clostridium difficile*, *Acinetobacter baumannii*, *Escherichia coli*, *Staphylococcus epidermidis, Pseudomonas aeruginosa, Klebsiella pneumoniae, Streptococcus pyogenes*, and different Mycobacterium species [34–37]. More recently, its germicidal properties have been tested on several types of viruses, such as IBV, IAV, HIV-1 and even SARS-CoV-2, with promising and encouraging results, although the mechanism of action in viruses is not yet fully understood [38–40].

In spite of the slower disinfection activity, a major advantage of this type of radiation over UVC is their far greater ability to penetrate through materials (e.g. transparent and opaque walls), allowing deeper disinfection of environments and less direct damage to irradiated surfaces.

The aim of this study was to evaluate the potential virucidal effect of violet-blue light, at different radiant exposures and stress conditions, against IDV.

2 Materials and methods

2.1 Influenza virus

Influenza D/bovine/Oklahoma/660/2013 (lineage D/660), kindly provided by Prof. Feng Li, University of Kentucky, was propagated in Madin-Darby Canine Kidney (MDCK) cells (ATCC-CCL-34) as previously reported [8].

2.2 Violet-blue light lamp

The violet-blue lamp prototype is composed by 9 LEDs 405 nm, 1.4 W each at 700 mA with the full width at half maximum (FWHM) of approx. 10–15 nm, positioned on a metal square support. The lamp system is supported by a metal scaffold, fixed to a metal squared base (30 cm × 30 cm), 32 cm from the lighting source, on which the outline of a multiwell plate has been traced to correctly carry out the tests (Fig. 1). The electromagnetic spectrum was characterised using an Avantes ULS2048CL EVO spectrophotometer (Avantes, Apeldoorn, Netherlands) and using the Avasoft measurement software (Avantes, Apeldoorn, The Netherlands). The energy map of the multi-well plate, measured at 32 cm from the LEDs, was used to determine the distribution of light irradiance. Light distribution on the surface was homogeneous, with a minimum difference of approximately 4% recorded between the side and central wells. The average irradiance recorded within the wells of the plate was 4 mW/cm^2.

2.3 Experimental method

Viral suspension of IDV was exposed to a violet-blue light (405 nm). The experiments were performed at different radiant exposures, calculated by multiplying the exposure time by the average irradiance on the wells: 5.4 J/cm^2 (22 min and 30 s), 10.8 J/cm^2 (45 min), 21.6 J/cm^2 (90 min), 43.2 J/cm^2 (180 min), and 86.4 J/cm^2 (360 min). For each time of exposure, the viral suspension was exposed to a different temperature (room temperature, 4 and 37 °C). Each time and temperature condition were tested in duplicate.

300 µl of viral suspension was placed in each of the 3 central wells (B2, B3 and B4) of a 24-well plate (Fig. 1) and exposed to the light for the defined times. For each exposure time and temperature, a virus control plate was placed under the same conditions but without radiation.

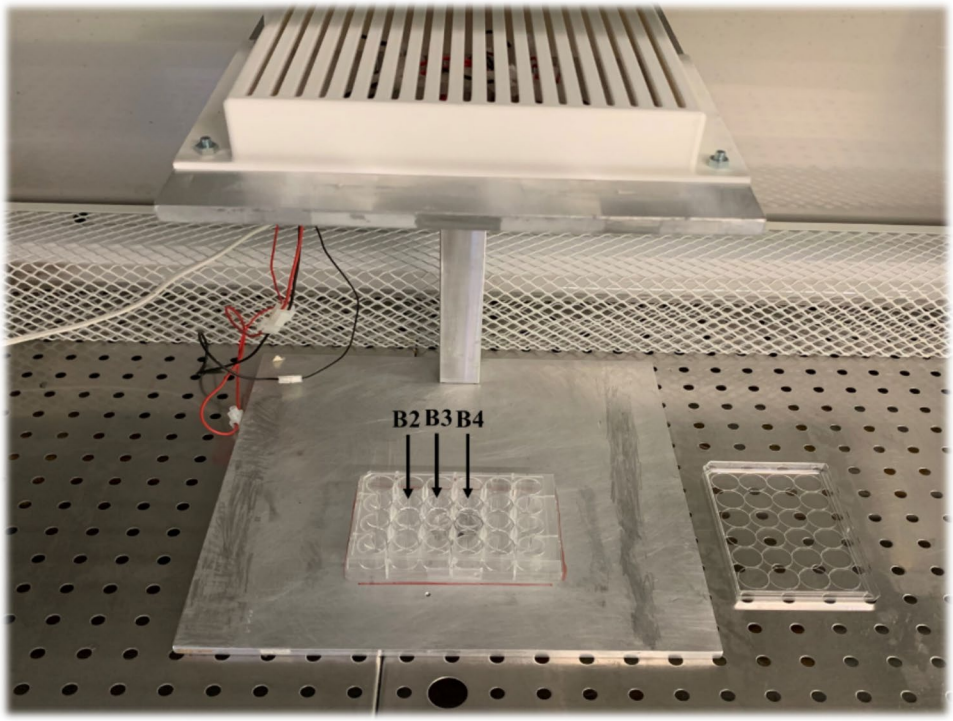


Fig.1 Violet-blue light lamp, the three central wells used for experiments are indicated as B2, B3 and B4. The homogeneous light distribution allowed all wells to be irradiated with an average irradiance of 4 mW/cm^2. The radiant exposure was calculated by multiplying the exposure time by the average irradiance. The inoculum was exposed to 5.4 J/cm^2, 10.8 J/cm^2, 21.6 J/cm^2, 43.2 J/cm^2, and 86.4 J/cm^2 respectively

At the end of exposure, virus titration was performed on MDCK cells. The MDCK cell cultures were grown at 37 °C in 5% CO_2 and pre-incubated on 96-well culture plates for 4 h. IDV was titrated in serial 1 $\log_{10}$ dilutions and 100 µl of each virus dilution were transferred to a plate containing 1.5×10^4 MDCK cells/well. After 96 h incubation at 37 °C 5% CO_2, plates were read for hemagglutination titre of the supernatant. 50 µl of supernatant of each dilution were transferred in a V-bottomed 96-well plate and incubated for 1 h with a 0.5% turkey red blood cells solution. The viral titre was reported as the reciprocal of the highest dilution showing haemagglutination, calculated using the Reed-Muench method [41] and expressed as $TCID_{50}$/ml.

To investigate the possible role of oxidative stress in the viral inactivation mechanism, the viral suspension was prepared with the addition of an antioxidant (N-acetylcysteine, NAC and superoxide dismutase, SOD) at different concentrations (0.5 M, 0.05 M, 0.005 M and 0.0005 M for NAC and 1 mg/ml, 0.1 mg/ml, 0.01 mg/ml and 0.001 mg/ml for SOD) and exposed to the lamp for 360 min at room temperature, together with a viral control without antioxidants. A no-exposure control was also performed for each of these conditions. Experiments were performed in duplicated, and results are expressed as mean of the tests.

2.4 Statistical analysis

Viral titre reduction after exposure was calculated with respect to virus control and expressed as $\log_{10}$ reduction. $\log_{10}$ reduction was calculated as difference between the viral titre of the virus control and the viral titre after exposure. $\log_{10}$ reduction was further converted to percent reduction, calculated as $\{1 - 1/[\text{power}(10,\ \log 10\text{reduction})]\} \times 100$.

For the assessment of possible role of oxidative stress, for each exposure condition (presence/absence of antioxidant and its concentration) the reduction in viral titre was calculated respectively to the same unexposed condition.

Statistical analysis was performed using Jamovi software ver. 2.3.21 (Jamovi, Sydney, Australia). ANCOVA analysis was performed taking a $p < 0.001$ as significant, with 'stress conditions' (to which the viral inocula were exposed) as the fixed factor, 'reduction in viral titre' as the dependent variable, and 'exposure times' at which the tests were performed as the main covariate. A post hoc Tukey test was then performed to show significant (and non-significant) comparisons between the different temperatures associated with exposure to blue-violet light (405 nm).

3 Results

The results showed that titre reduction of IDV was dependent on time and temperature of exposure.

Viral titres decreased proportionally to the exposure time. The maximum reduction of the viral titre (2.293 $\log_{10}$; 99.5%), was obtained after exposure of IDV at room temperature and at 86.4 J/cm^2. By reducing the radiant exposure to the violet-blue light lamp at room temperature, there is

less reduction in viral titre, more specifically: 1.375 $\log_{10}$ (95.8%) at 43.2 J/cm^2, 0.940 $\log_{10}$ (88.5%) at 21.6 J/cm^2, 0.668 $\log_{10}$ (78.5%) at 10.8 J/cm^2 and 0.228 $\log_{10}$ (40.8%) at 5.4 J/cm^2 (Fig. 2 and Table 1).

As for room temperature, temperatures of exposure of 4 and 37 °C showed the greater reduction of the viral titre at 86.4 J/cm^2: 1.397 $\log_{10}$ (96.0%) for 4 °C and 1.747 $\log_{10}$ (98.2%) for 37 °C. Decreasing the radiant exposure to the violet-blue light lamp led to a less reduction of viral titre, as observed for the exposure to room temperature (Table 1).

Comparing the exposure temperatures, the reduction of viral titre of IDV was found significantly higher for room temperature than for 4 and 37 °C temperatures ($p < 0.001$) (Table 2).

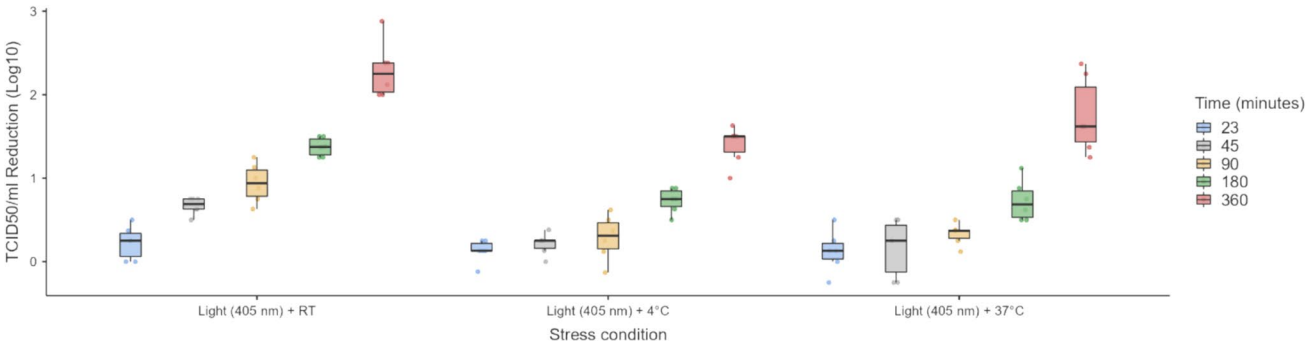


Fig. 2 Boxplot of the mean $\log_{10}$ reduction of IDV after exposure to blue-violet light (405 nm) by time and temperature of exposure. The whiskers correspond to the minimum and maximum values of each exposure condition, while the median value is represented by the line within each box plot. Room temperature, RT

Table 1 Mean $\log_{10}$ reduction of IDV after exposure to blue-violet light (405 nm) at different temperatures and by radiant exposures. Average irradiance of 4 mW/cm^2 on the multiwell plate. Standard error, 95% confidence intervals were reported. Room temperature, RT;. Lower Confidence Interval, Low.; Upper Confidence Interval, Up

Stress condition	Radiant exposure (J/cm^2)	$TCID_{50}$/mL reduction (Log_{10})	Standard error	95% confidence interval	
				Low	Up
Light (40 nm) + RT	5.4	0.228	0.081	0.069	0.388
	10.8	0.668	0.041	0.587	0.749
	21.6	0.940	0.095	0.754	1.126
	43.2	1.375	0.046	1.286	1.464
	86.4	2.293	0.137	2.025	2.561
Light (405 nm) + 4 °C	5.4	0.128	0.055	0.020	0.236
	10.8	0.210	0.053	0.106	0.314
	21.6	0.288	0.110	0.072	0.505
	43.2	0.732	0.060	0.614	0.850
	86.4	1.397	0.094	1.212	1.581
Light (405 nm) + 37 °C	5.4	0.127	0.102	− 0.073	0.327
	10.8	0.167	0.139	− 0.107	0.440
	21.6	0.332	0.053	0.227	0.436
	43.2	0.728	0.099	0.535	0.922
	86.4	1.747	0.188	1.378	2.116

Table 2 Tukey post-hoc analysis for comparison between exposure type related to temperatures

Comparison*			Mean difference	P_{tukey}
Stress condition		Stress condition		
Light (405 nm) + RT	–	Light (405 nm) + 4 °C	0.55	< 0.001
	–	Light (405 nm) + 37 °C	0.481	< 0.001
Light (405 nm) + 4°C	–	Light (405 nm) + 37 °C	-0.069	0.558

* Comparisons are based on estimated marginal mean

Viral titre of virus control showed to be stable up to 360 min' regardless temperature (data not shown).

The results from lamp exposure in the presence of antioxidants showed that the effect of the lamp is at least partly counteracted by the presence of NAC (Table 3). The highest concentrations of NAC were found cytotoxic, and the viral titre was not calculated. However, at 0.005 M no reduction in viral titre was observed in the lamp-exposed virus compared to the unexposed virus control, and at 0.0005 M the reduction in viral titre was of 68.4% compared to the 99.2% reduction observed after 360 min of exposure at room temperature in the absence of NAC. On the other hand, SOD showed no protective effect in counteracting the effects of the lamp exposure on the virus.

4 Discussion

Following its first isolation in 2011 from swine exhibiting influenza-like illness, IDV has been isolated in other animal species worldwide. IDV infection causes respiratory diseases in cattle resulting in possible loss, including costs, for herds. Moreover, it poses a threat not only to animal health but also to humans [12]. Indeed, antibodies against IDV were detected in occupational workers in contact with cattle in USA [9] but also in veterinarians in Italy [11], suggesting possible infection in humans. Active surveillance activities in hospitals and at airports reported molecular detection of IDV in environmental air samples [42, 43], and IDV was also detected in a nasal wash of a pig worker in Malaysia [44].

Photodynamic inactivation is an emerging alternative for the treatment and control of viral infections, representing an effective way to minimise the spread of disease by inactivating and reducing the number of viral particles in the environment. The germicidal properties of photodynamic inactivation have been tested on several types of viruses, including influenza viruses and SARS-CoV-2 [45–47].

In this study we evaluated the potential inactivation of IDV exposed to a violet-blue light lamp. IDV was exposed up to 360 min (86.4 J/cm^2) at different temperatures, showing a reduction in the viral titre proportional to the increase of the radiant exposure. At 86.4 J/cm^2, we observed a viral titre reduction above 95% for all the temperatures tested, reaching the higher value (99.5%) at room temperature.

As reported for other viruses [40, 48], the virucidal effect of the lamp was proportional to the radiant exposure. However, differences were observed among temperatures of exposure. The greatest virucidal effect was in fact observed at room temperature, while at 37 and 4 °C the effect seemed to be somehow reduced. At least for 4 °C, a possible explanation might be that low temperatures have a protective effect on viruses, as they are usually preserved at 4 °C or − 80 °C.

In previous studies, IDV showed to be resistant to high temperatures and low pH [1, 15, 49]. Our results from virus control (not exposed to the lamp) confirm that IDV is stable up to 360 min in a range of temperatures from 4 to 37 °C, probably due to its viral structure, and in particular the HEF protein, that makes the virus particularly resistant [15]. Also, there could be other protective effects on the virus, such as increased humidity, which would have had a shielding effect with the radiation, reducing it slightly less than the virus at RT and ambient humidity.

In addition, experiments with viruses exposed to light in the presence of antioxidants have shown that oxidative damage to the lipid envelope and surface proteins is likely the main cause of virus inactivation. As widely reported in

Table 3 Mean $\log_{10}$ reduction of IDV after exposure to blue-violet light (405 nm) with the addition of an antioxidant (N-acetyl-L-cysteine, NAC and superoxide dismutase, SOD) at different concentrations

Antioxidant	Antioxidant concentration	$\log_{10}$ $TCID_{50}$/ml control virus	$\log_{10}$ $TCID_{50}$/ml treated virus (405 nm light)	$\log_{10}$ $TCID_{50}$/ml reduction	% reduction of the virus titer
NAC	0 M*	5.75	3.63	2.12	99.2%
	0.0005 M	4.75	4.25	**0.50**	**68.4%**
	0.005 M	5.00	5.00	**0.00**	**0.0%**
	0.05 M	NA	NA	NA	NA
	0.5 M	NA	NA	NA	NA
SOD	0 mg/ml*	5.75	3.63	2.12	99.2%
	0.001 mg/ml	5.00	3.50	1.50	96.8%
	0.01 mg/ml	5.25	3.50	1.75	98.2%
	0.1 mg/ml	4.75	3.25	1.50	96.8%
	1 mg/ml	4.75	2.50	2.25	99.4%

Percentage reduction is also reported

*The concentration of 0 M and 0 mg/ml corresponds to the treatment of the virus with light at 405 nm in the total absence of antioxidant

NA, not applicable

the literature, IAV and IBV are particularly resistant to intracellular oxidative stress upon virus entry into the host cell [50, 51]. However, studies showing a similar assessment are still lacking and limited in number for IDV. It is, therefore, possible that the virus shows an increased susceptibility to oxidative damage and that this is due to the phenomenon we studied with 405 nm light. Although in different contexts, ROS generated during the infectious process inside the host cell and ROS generated in response to exposure to a specific light frequency by photo-excitable molecules can damage vital structures of the virus, such as the phospholipid membrane, and affect its replication cycle. This type of frequency allows the photoexcitation of photosensitive molecules that can generate ROS [52], but also induce protein degradation and membrane lipid autoxidation [53]. Moreover, the degradation of the viral membrane proteins is another consideration. While IAV and IBV have two cell adhesion glycoproteins (HA and NA) that bind to host cell receptors and initiate viral entry, ICV and IDV have only one core protein, the HEF [14, 54]. It is, therefore, possible that impairment of this single protein, caused by oxidative damage induced by protein photodegradation, may affect the ability of the virus to adhere to the host cell membrane and complete its replication cycle.

This study has limitation. The close environment in which the experiments were conducted (a closed laboratory with doors and windows closed) excludes some variables, such as air circulation in the room. Moreover, experiments were performed in liquid medium instead of aerosolized droplets, that better resemble the airborne transmission. Prolonged exposure to blue light may increase the temperature of the samples. The temperature of the sample at the end of exposure was not measured. The contribution of the heat of the sample in the inactivation of the virus warrants further investigations.

As previously reported for bacteria [55], 405 nm light has virucidal effect on IDV, especially on prolonged exposure. Virucidal activity of similar lights were already reported for other respiratory viruses, such as adenovirus, respiratory syncytial virus and SARS-CoV-2 [16, 48, 56], as well as IAV [40]. To our knowledge, this is the first study demonstrating a significant decrease in the infectivity of IDV after exposure to a 405 nm visible light.

In a previous study [48], mucins and other organic substances contained in respiratory swabs or droplets were indicated as possible photosensitizers, allowing oxidation of the molecules and damaging the virus itself. In this study, the evaluation of the possible role of oxidative stress in the mechanism of viral inactivation was carried out on viral propagates in cell culture. The results showed that the effect of the lamp is at least partly counteracted by the presence of antioxidants. This contributes to the idea that not only substances present in the medium in which the virus is suspended, but also components of the virus itself, may act as photosensitisers, suggesting a possible process of, i.e. lipid peroxidation by the viral envelope component. However, it is important to note that not all antioxidants were shown to have a photoprotective effect on the virus. In fact, for SOD, no different results were found than when the virus was exposed to 405 nm radiation. This may be due to the fact that the radicals formed during exposure were not inactivated by the antioxidant, since radiation produces superoxide in hydrogen peroxide solutions and hydroxyl radicals and solvated electrons in deoxygenated water [57].

It is more likely that in this case the radicals formed by prolonged exposure were singlet oxygen, ozone and hydrogen peroxide, which caused the peroxidation of the lipids.

Given that the virucidal action of blue light may induce oxidative damage to the lipid envelope, it may be interesting to continue the investigation by testing lipophilic antioxidants to determine whether they have a protective effect during the inactivation process.

Inactivation of airborne viruses, such as influenza viruses, is crucial to prevent infections. IDV has the second widest host range after IAV [2, 58]. As reported in this study, 405 nm light could be used to reduce IDV spread in potentially hotspot areas for animals and humans, as integration to classical cleaning and disinfection methods. Indeed, the findings of this research indicate the potential of 405 nm light as a non-invasive method for inactivating airborne viruses. This wavelength is a potential approach for targeted inactivation due to its oxidative vulnerability to both lipids and viral proteins. Unlike traditional antiviral drugs, which often target viral replication or enzymatic activity, 405 nm light induces structural damage via oxidative stress, removing the necessity for viral entrance into host cells [59, 60]. This strategy might be especially effective in settings where direct viral contact is inevitable, such as healthcare facilities or air filtration systems [61].

Despite its intriguing premise, further study is required to completely understand of 405 nm light's effectiveness and long-term safety. Also, studying how this radiation and antioxidant therapy work together might help us figure out the best balance between their ability to inactivate viruses and their ability to control oxidative stress. This could lead to safer and more effective use in both therapeutic and environmental settings.

Author contributions Conceptualization: CMT; formal analysis: DA; funding acquisition: CMT; methodology: SM; investigation: BP and SM; project administration: SM; resources: GM, EM, and CMT; visualisation: BP; supervision: CMT; writing—original draft preparation: BP, SM, and DA; writing—review and editing: ID, GM, EM, and CMT.

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Data availability The original contributions presented in the study are included in the article, and further inquiries can be directed to the corresponding author.

Declarations

Competing interests EM is founder and Chief Scientific Officer of VisMederi srl and VisMederi Research srl.

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