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# Methylene blue photochemical treatment as a reliable SARS-CoV-2 plasma virus inactivation method for blood safety and convalescent plasma therapy for COVID-19

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## Abstract

**Background:** In 2020, a new coronavirus, SARS-CoV-2, quickly spread worldwide in a few months. Although coronaviruses typically infect the upper or lower respiratory tract, the virus RNA can be detected in plasma. The risk of transmitting coronavirus via transfusion of blood products remains. As more asymptomatic infections are identified in COVID-19 cases, blood safety has become particularly important. Methylene blue (MB) photochemical technology has been proven to inactivate lipid-enveloped viruses with high efficiency and safety. The present study aimed to investigate the SARS-CoV-2 inactivation effects of MB in plasma.

**Methods:** The SARS-CoV-2 virus strain was isolated from Zhejiang University. The live virus was harvested from cultured VERO-E6 cells, and mixed with MB in plasma. The MB final concentrations were 0, 1, 2, and 4  $\mu\text{M}$ . The “BX-1 AIDS treatment instrument” was used at room temperature, the illumination adjusted to  $55,000 \pm 0.5$  million Lux, and the plasma was irradiated for 0, 2, 5, 10, 20, and 30 mins using light at a single wavelength of 630 nm. Virus load changes were measured using quantitative reverse transcription-PCR.

**Results:** BX-1 could effectively eliminate SARS-CoV-2 within 2 mins in plasma, and the virus titer declined to 4.5 log<sub>10</sub> TCID<sub>50</sub> (median tissue culture infectious dose)/mL.

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**Conclusion:** BX-1 is based on MB photochemical technology, which was designed to inactivate HIV-1 virus in plasma. It was proven to be safe and reliable in clinical trials of HIV treatment. In this study, we showed that BX-1 could also be applied to inactivate SARS-CoV-2. During the current outbreak, this technique it has great potential for ensuring the safety of blood transfusions, for plasma transfusion therapy in recovering patients, and for preparing inactivated vaccines.

**Keywords:** Methylene blue, Photochemical treatment, SARS-CoV-2, Plasma virus inactivation, Blood safety, Convalescent plasma therapy, COVID-19

## Background

Corona virus disease 2019 (COVID-19) is caused by severe acute respiratory syndrome (SARS)-corona virus (CoV)-2. The first outbreak occurred at the end of 2019, causing symptoms such as fever and pneumonia. In serious cases, the patient might die. As of the early March 2021, the COVID-19 epidemic had appeared in all continents except Antarctica, about 117 million people have been diagnosed worldwide, and the death toll has approached 2.6 million, seriously threatening global public health [1].

SARS-CoV-2 was first isolated from epithelial cells of the human airway, and was identified by next-generation sequencing technology in January 2020 as a new member of the beta coronaviruses ( $\beta$ -CoVs) [2]. Coronaviruses are enveloped, single-stranded RNA viruses [2]. From 2002 to 2003, more than 8000 patients were infected by SARS-CoV, which causes SARS, and the World Health Organization (WHO) finally reported 774 related deaths. Since September 2012, a total of 2494 laboratory-confirmed Middle East respiratory syndrome (MERS)-CoV infected cases have been reported. The WHO has reported 858 MERS-CoV-related deaths [3, 4]. These emerging infectious diseases with global transmission are caused by  $\beta$  CoVs.

Although coronaviruses cause respiratory tract infections, several studies have shown that the viral RNA of SARS-CoV [5], MERS-CoV [6], or SARS-CoV-2 [7, 8] can be detected in blood plasma. As early as 2003, the WHO noted that blood products could be contaminated with SARS-CoV. Although no cases of SARS-CoV infection caused by blood transfusion have been reported, there is still a theoretical risk of transmitting SARS-CoV or other coronaviruses through blood transfusion [9]. As more asymptomatic infections are identified among COVID-19 cases [10], blood safety becomes more important [11, 12]. In addition, there are currently no specific drugs or vaccines that are effective against SARS-CoV-2. Patient convalescent plasma therapy could be a rapid and effective treatment for this epidemic situation [13]. However, the critical aspect of this method is to ensure the complete inactivation of SARS-CoV-2 in the

plasma from donors. There is an urgent need for a reliable and practical method of plasma SARS-CoV-2 inactivation.

Photochemical treatment methods are used as pathogen inactivation/reduction technologies (PRTs). Photochemical methods to kill viruses are performed by adding methylene blue (MB) into blood products followed by visible light treatment. Methylene blue is a photosensitizer with a maximum absorption peak of 670 nm. It is usually used as an antidote to treat methemoglobinemia and cyanide poisoning caused by nitrite poisoning. The surface of MB carries a positive charge. It can be embedded into DNA or RNA, especially in combination with negatively charged G-C base pairs of viral nucleic acids. Under visible light, MB absorbs light energy, activates it from the ground state to a singlet state, and generates singlet oxygen through electron transfer, which damages and breaks nucleic acids to kill viruses. Viral envelopes and nucleic acids can be targets of MB attack. Alamdari and colleagues used leucomethylene blue, a reduced form of MB, as a therapeutic method for COVID-19 infection [14]. Furthermore, they used a combination of MB-vitamin C-N-acetyl Cysteine to treat five patients with COVID-19 and four out of five survived [15]. The possibility of using MB for either the prevention of viral infection or the development of photoactive fabrics has been proposed by Almeida et al. [16]. Under light, MB can cause nucleic acid strand breaks [17]. The visible light source can be a halogen lamp, a metal halide lamp, or a fluorescent lamp, with full-band white light. However, because of the low excitation efficiency of MB in full-band white light, this process also generates heat to destroy plasma components. The 'BX-1 AIDS treatment instrument', developed by Boxin Biotechnology Development LTD (Beijing, China), uses LED single wavelength light combined with MB and is highly effective at killing various lipid enveloped viruses, such as HIV-1 and other viruses [18, 19]. As SARS-CoV-2 is a new type of coronavirus, its tolerance to physicochemical conditions is unclear. Therefore, in the present study, the 'BX-1 AIDS treatment instrument' was used to study the plasma inactivation of a SARS-CoV-2 isolate from Zhejiang University.

## Methods

### Cells, instruments, reagents, and viruses

VERO-E6 cells bought from ATCC (Manassas, VA, USA; Cat. (ATCC® CRL-1586™) were stored in the laboratory of Zhejiang University. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin (P/S), and phosphate-buffered saline were bought from GIBCO (Grand Island, NY, USA). MB is a product of Jichuan Pharmaceutical Group Co., Ltd. (Taixin, China). Human plasma was donated by volunteers, and written informed consents were obtained. All experimental operations involving live viruses were carried out in Zhejiang University's Biosafety Level 3 (BSL-3) Laboratory. The research protocol was approved by the Ethics Committee of the First Affiliated Hospital of Zhejiang University School of Medicine.

### Methylene blue-light inactivated virus

To 180 mL of healthy human plasma, 20 mL of live SARS-CoV-2 spike virus was added. Diluted MB was added to 200 mL of the plasma-virus mixture and mixed to a final MB concentration of 1, 2, or 4  $\mu$ M. The "BX-1 AIDS treatment instrument" was used at room temperature, and illumination was adjusted to  $55,000 \pm 0.5$  million Lux. The plasma samples were irradiated for 0, 2, 5, 10, 20, or 40 mins under light at a single wavelength of 630 nm. About 1 mL of plasma was taken at each time point, diluted 10-fold with DMEM medium containing 2% FBS, and filtered through a 0.45- $\mu$ m filter (Millipore, Billerica, MA, USA) for virus titer detection. At the same time, a virus control was set (only virus in the plasma, left at room temperature for 40 mins as an untreated control), a control with pure MB was set (virus and 4  $\mu$ M MB added to the plasma, with no light treatment, and allowed to stand for 40 mins as a control for the effect of MB on the virus), and finally a light-only control was set (only the virus was added to the plasma, and light was used for 40 mins as a control of the effect of light on the virus). This experiment was repeated three times.

### Virus titer measurement

After trypsinization of VERO-E6 cells,  $1 \times 10^4$  cells/well were inoculated into 96-well cell culture plates in 100  $\mu$ L of culture medium per well. After the cells grew into a single layer in the 96-well plate, the culture medium was discarded and the treated plasma was seeded into the wells. The treated plasma was log-diluted with 2% FBS in DMEM medium from  $10^2$ – $10^7$  times. This process was repeated for 4 wells per dilution, 200  $\mu$ L/well. Normal cell control wells were established (with cells, virus-free). The 96-well plate was placed in an incubator with 5% CO<sub>2</sub> at 37 °C. After 3 h of incubation, the supernatant was washed

off, and 200  $\mu$ L /well of 2% FBS DMEM medium was added. Cell lesions were observed every 24 h until 6 d. The TCID<sub>50</sub> (median tissue culture infectious dose) was calculated according to the Reed-Muench method. The cell culture supernatant at 6 d of the culture was pipetted, and the viral nucleic acid load was measured. This experiment was repeated three times.

### Three generations of blind tests

One milliliter of the test plasma with 1, 2, and 4  $\mu$ M MB was irradiated for 40 min. It was then diluted 10 times with 2% FBS DMEM medium, filtered through a 0.45- $\mu$ m filter, and added to VERO-E6 cells. After 48 h, 1 mL of the supernatant of the culture was diluted 10 times with 2% FBS DMEM medium, and added to VERO-E6 cells for three passages. These cells were observed for cytopathic effects. For the duration of the 3rd generation of cell blind transmission, the sample was deemed positive (+) if cytopathic lesions appeared at any time, indicating that the virus was not completely inactivated; samples were deemed negative (-) if no cytopathic lesions appeared, indicating that the virus had been completely inactivated. This experiment was repeated three times.

### Viral load of culture supernatant measured using quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

**Nucleic acid extraction:** Using 200  $\mu$ L of virus culture supernatant, the MVR01 magnetic bead method nucleic acid extraction kit was used (article number: ZM-0044, Shanghai Zhijiang Biotechnology Co., Ltd., Shanghai, China) in an EX2400 automatic nucleic acid extraction instrument (Shanghai Zhijiang Biotechnology Co., Ltd). Virus RNA was extracted, and the elution volume was about 50  $\mu$ L.

**qRT-PCR:** A new coronavirus nucleic acid assay kit (Cat. No. Z-RR-0479-02-50, Shanghai Zhijiang Biotechnology Co., Ltd.) was used for qRT-PCR to detect the viral load, following the manufacturer's instructions. Briefly, the qRT-PCR amplification reaction tube was placed on a LightCycler® 480II (Roche, Basel, Switzerland) qPCR instrument, and FAM and VIC (or TEXAS RED) fluorescence channels were selected for detection. The recommended cycle parameter settings were: 45 °C for 10 min and 95 °C for 15 min; then 45 cycles of 95 °C for 15 s and 60 °C for 60 s; with single-point fluorescence detection at 60 °C. A cycle threshold (CT) value below 35 was considered effective amplification, and a CT value above 35 was considered undetected.

### Statistical analysis

The statistical analyzes were performed using SPSS 20.0 (IBM Corp., Armonk, NY USA). Student's t-test was

employed if the measurement data exhibited a normal distribution, and non-parametric test if it did not exhibit a normal distribution.  $P < 0.05$  was considered to indicate statistical significance.

## Results

### Effect of SARS-CoV-2 infection on VERO-E6 cells

SARS-CoV-2 (100  $\mu$ L of 4 log<sub>10</sub> TCID<sub>50</sub>/mL) was seeded into a 96-well plate with monolayers of VERO-E6 cells and cultured to observe the CPE. SARS-CoV-2 had a strong CPE, and the infected cells showed obvious pathological necrosis and large-scale detachment (Fig. 1).

### BX-1-MB photochemical method inactivation of SARS-CoV-2

SARS-CoV-2 (100  $\mu$ L of 4 log<sub>10</sub> TCID<sub>50</sub>/mL) was seeded into a 96-well plate with a single layer of VERO-E6 cells, and 1  $\mu$ M (Fig. 1a), 2  $\mu$ M (Fig. 1b), or 4  $\mu$ M of MB (Fig. 1c) was added. Blue light was applied for 40 mins. The control groups were the virus only (Fig. 1d), MB plus no illumination group (Fig. 1e) and the virus-only illuminated group (Fig. 1f). All treatments were cultured and observed for the CPE. 1  $\mu$ M, 2  $\mu$ M, and 4  $\mu$ M of MB could completely inactivate the virus, and the inactivated virus had no effect on cell growth. The non-illuminated group (Fig. 1e) and the virus-only illuminated group (Fig. 1f) were unable to inactivate the virus, and the infected cells showed significant lesion, necrosis, and large-scale exfoliation.

In the process of three passages of cell blind transmission, no cell lesions appeared in any generation of cells, indicating that the virus had been completely inactivated. The qPCR results indicated that virus-containing plasma treated with 1, 2, and 4  $\mu$ M of MB and light could completely inactivate the virus in 2 min (Table 1).

### Viral titer detection after application of the BX-1 MB photochemical method to inactivate SARS-CoV-2

Using the CPE method, we found that the BX-1 MB photochemical method could inactivate SARS-CoV-2 in 40 mins. To further study the inactivation efficiency, we detected the viral titer of BX-1 MB photochemical-treated virus plasma at 0, 2, 5, 10, 20, and 40 min. Viral titer tests found that virus plasma containing 1  $\mu$ M and 2  $\mu$ M MB had a virus reduction  $> 4$  Log<sub>10</sub> TCID<sub>50</sub> for 2 mins; the group with 4  $\mu$ M MB had an initial titer because differences between batches in the test (Fig. 2).

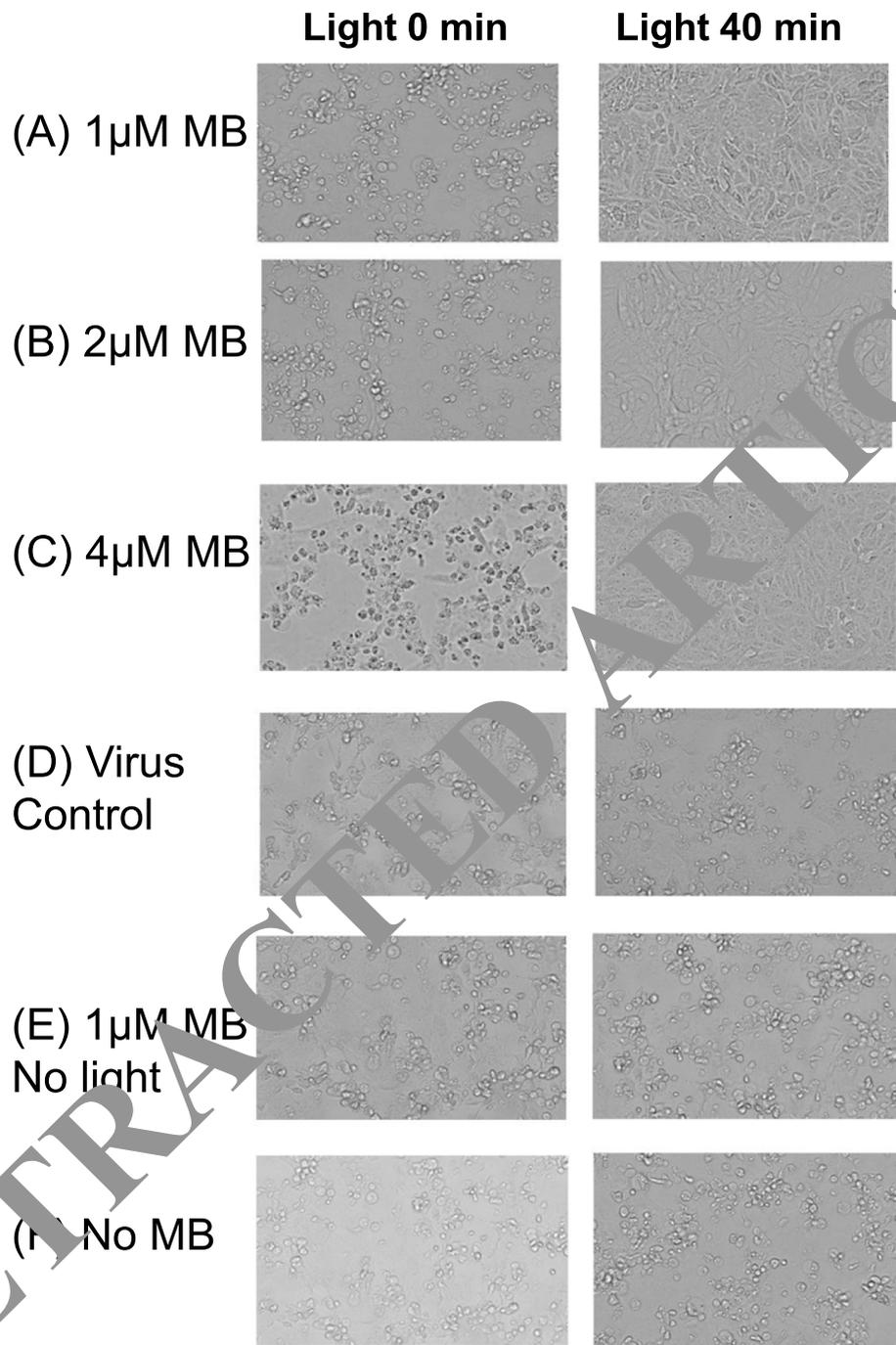
## Discussion

The new coronavirus SARS-CoV-2, which appeared in December 2019, spread globally within 3 months and is being monitored closely worldwide. The following are safety considerations for blood transfusions or blood

products: (1) Viral RNA in plasma or serum can be detected in patients with COVID-19 2 or 3 d before the onset of symptoms [8]; (2) most patients, especially young adults who can donate blood, experience milder symptoms than do the elderly; (3) multiple asymptomatic carriers have been found in China and other countries, which increases the possibility of blood donation by COVID-19 or virus carriers [10]; (4) infection rates of patients in the incubation period are still uncertain, and there is yet no data on viral load in plasma, serum, or lymphocytes during the incubation period. Blood transfusions carry the risk of transmitting SARS-CoV-2, and thus measures to inactivate pathogens in blood products should be explored as soon as possible.

Coronaviruses are enveloped, single-stranded RNA viruses. Previous studies indicated that coronaviruses are generally susceptible to acid, alkaline, and heat [20]. After the outbreak of SARS and MERS, some studies investigated the potential of PRTs to reduce or completely eliminate the potential risks of coronavirus transmission through blood products or derivatives [21]. Research on plasma inactivation methods has focused on heat and photochemical treatment methods. (1) Heat method, in general, 30 mins at 60 °C is sufficient to reduce SARS-CoV from cell-free plasma [22], and 25 mins at 56 °C reduced MERS virus by more than 4 log<sub>10</sub> TCID<sub>50</sub>/mL [23]. Heating can denature proteins in blood products; therefore, it cannot be used to manufacture plasma-derived products. (2) Photochemical treatment methods. Different wavelengths of light affect the viability of SARS and MERS viruses in the blood. UV light, Amotosalen, or riboflavin can inactivate pathogenic nucleic acids [22]. Keil et al. performed SARS-CoV-2 inactivation of blood products in standard blood bags using the Riboflavin and the UV method and the data indicated that the reductions of the viral titres were  $\geq 3.40$  Logs and  $\geq 4.53$  Logs in plasma and platelets, respectively [24], which are on the same order of magnitude as our data. It has been previously reported that MB plus visible light is capable of inactivating coronavirus in plasma [21, 25]. Previously, the prototype 'plasma virus inactivator' JY-1 developed by Boxin (Beijing) Biotechnology Development LTD, under the condition of a single-wavelength LED illumination of 40,000 lx, was used for treatment together with 1  $\mu$ M MB for 30 min [26, 27]. The new BX-1 'AIDS treatment instrument' used in this study can reduce the titer of new coronavirus SARS-CoV-2 by 4.5 log<sub>10</sub> TCID<sub>50</sub>/mL in 2 mins (Fig. 2), which is a strong advantage for inactivation of viruses in plasma.

MB and LED based BX-1 technology provides a new approach for inactivation of SARS-CoV-2 in plasma, which may be useful for convalescent plasma therapy of critical ill patients with COVID-19. Convalescent plasma therapy is a rapid and effective treatment that has been



**Fig. 1** E6 cells infected by SARS-CoV-2 across different treatment groups (100  $\times$ ). To 180 mL of healthy human plasma, 20 mL of indicator virus was added at a 9: 1 (V: V) ratio, and mixed. Methylene blue (MB) was added to 200 mL of plasma virus mixture and mixed to a final concentration of 1  $\mu$ M (a), 2  $\mu$ M (b), or 4 (c)  $\mu$ M. The "BX-1 AIDS treatment instrument" was used at room temperature, the illumination adjusted to 55,000  $\pm$  0.5 million Lux, and irradiation was carried out for 40 mins under single wavelength 630-nm light. The virus control (d) was set (only virus in the plasma, left at room temperature for 40 mins as an untreated control), and control with pure 1  $\mu$ M MB (e) was set (virus and 1  $\mu$ M MB added to the plasma, with no light treatment, and allowed to stand for 40 mins as a control for the effect of MB on the virus). Finally, the light-only control (f) was set (only the virus was added to the plasma, and light was used for 40 mins as a control of the effect of light on virus). This experiment was repeated three times

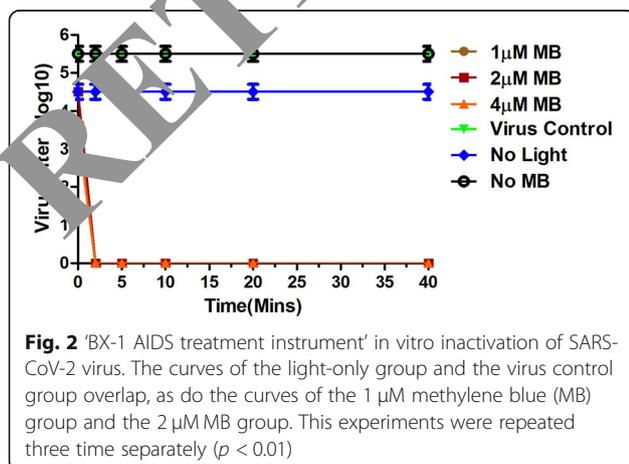
**Table 1** Plasma SARS-CoV-2 virus titration test, with cycle threshold (CT) dilution of supernatant and nucleic acid qRT-PCR detection

	Light time (Min)	Virus Dilutions (times)					
		10 <sup>2</sup>	10 <sup>3</sup>	10 <sup>4</sup>	10 <sup>5</sup>	10 <sup>6</sup>	10 <sup>7</sup>
1µM MB CT	0	13.41	13.98	15.92	18.74	ND	ND
	2	ND	ND	ND	ND	ND	ND
	5	ND	ND	ND	/	/	/
	10	ND	ND	ND	/	/	/
	20	/	/	/	/	/	/
2µM MB CT	0	12.66	12.97	15.32	17.79	ND	ND
	2	ND	ND	ND	ND	ND	ND
	5	ND	ND	ND	/	/	/
	10	ND	ND	ND	/	/	/
	20	/	/	/	/	/	/
4µM MB CT	0	13.31	13.75	16.86	ND	ND	ND
	2	ND	ND	ND	ND	ND	ND
	5	ND	ND	ND	/	/	/
	10	ND	ND	ND	/	/	/
	20	/	/	/	/	/	/
Virus Control CT	0	13.03	12.94	14.72	16.27	19.56	21.68
	40	13.94	14.17	16.27	15.75	19.56	ND
No MB CT	0	12.88	12.97	13.71	18.71	24.53	30.92
	40	13.15	13.41	15.51	16.6	21.94	ND
1µM MB No Light CT	0	13.76	12.42	15.51	16.62	ND	ND
	40	13.3	13.44	14.36	14.22	ND	ND

Note: '/' indicates not applied. ND indicates not detected. This experiment was repeated three times

applied for severe infectious diseases for over 100 years [13]. In 1890, Emil von Behring from Germany and Saburo Kitari from Japan announced an important discovery: They kept injecting small amounts of non-lethal tetanus into animals, which produced an antitoxin in the blood. This could neutralize the bacillus toxicity of tetanus injected into the body. The serum could also be isolated from animals that have acquired tetanus immunity and injected into other animals to enhance their immunity to tetanus. For this, Behring won the 1901 Nobel Prize in Medicine for his research on diphtheria serum therapy. The earliest application of convalescent plasma

therapy allowed humans to overcome diphtheria [13]. In 2014, during Ebola virus disease outbreaks, the WHO recommended the use of convalescent plasma as an empirical treatment [28]. Previously, in the clinical treatment of SARS coronavirus, doctors in Hong Kong, Taiwan, and Singapore had shown that convalescent plasma therapy could improve the conditions for critical ill patients with SARS for whom comprehensive treatment had not been effective [29]. Given this long history of serum-based treatment in the modern era, and its established safety and efficacy, convalescent plasma transfusion should be used to treat patients with COVID-19 [30].



**Fig. 2** 'BX-1 AIDS treatment instrument' in vitro inactivation of SARS-CoV-2 virus. The curves of the light-only group and the virus control group overlap, as do the curves of the 1 µM methylene blue (MB) group and the 2 µM MB group. This experiments were repeated three time separately ( $p < 0.01$ )

**Conclusion**

BX-1 based MB photochemical technology could inactivate SARS-CoV-2 efficiently and has great potential for ensuring the safety of blood transfusions, for plasma transfusion therapy in recovering patients, and for preparing inactivated vaccines.

**Abbreviations**

BSL-3: Biosafety Levels 3; COVID-19: Coronavirus disease outbreak 2019; CPE: Cytopathic effect; LED: Light-emitting diode; MB: Methylene blue; MERS-CoV: Middle East respiratory syndrome coronavirus; PRTs: Pathogen inactivation/reduction technologies; SARS: Severe acute respiratory syndrome; SARS-CoV-2: Severe acute respiratory syndrome coronavirus type 2; UV: Ultraviolet; WHO: World Health Organization

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## Authors' contributions

CJ, BY, MJ, and NW conceived and designed the study and took responsibility for the integrity of the data and the accuracy of the data analysis. CJ, HY, FL, LC, and XL performed the basic experiments and collected the data. CJ and BY processed the statistical data. CJ, JZ, XZ, HW, and BY drafted the manuscript. BY, MJ, and NW revised the final manuscript. All authors contributed to the data analysis and approved the final version.

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## Availability of data and materials

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

## Declarations

### Ethics approval and consent to participate

The study was approved by the Ethics Committee of the First Affiliated Hospital of Zhejiang University School of Medicine (Reference No. 2020-HIT-054). Written informed consents were obtained from the plasma donors.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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