

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

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Methylene blue, Photochemical treatment, SARS-CoV-2, Plasma virus inactivation, Blood safety and convalescent plasma therapy, COVID-19 outbreak

Abstract

Background

With the outbreak of unknown pneumonia in Wuhan, China in December 2019, a new coronavirus (SARS-CoV-2) attracted worldwide attention. Although coronaviruses typically infect the upper or lower respiratory tract, discovery of the virus in plasma is common. Therefore, the risk of transmitting coronavirus through transfusion of blood products remains. As more asymptomatic infections are found in COVID-19 cases, blood safety is shown to be particularly important, especially in endemic areas.

Study Design and Methods

BX-1, an 'AIDS treatment instrument' based on methylene blue (MB) photochemical technology, developed by Boxin (Beijing) Biotechnology Development LTD, has proven that inactivation of lipid-enveloped viruses such as HIV-1 in plasma has high efficiency, without damage to other components in the plasma, and proved safe and reliable in clinical trials of HIV treatment. In order to confirm the inactivation effect of BX-1 in SARS-CoV-2, we used the SARS-CoV-2 virus strain isolated from Zhejiang University for plasma virus inactivation studies.

Results and Conclusion

BX-1 can effectively eliminate SARS-CoV-2 within 2 mins, and the virus titer decline can reach 4.5 log₁₀ TCID₅₀/mL. Faced with the expanding epidemic, BX-1 is safe for blood transfusion and plasma transfusion therapy in recovery patients, and the inactivated vaccine preparation has great potential for treatment in the current outbreak.

Introduction

Coronavirus disease outbreak 2019 (COVID-19) is caused by severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2). The first outbreak occurred at the end of 2019, causing symptoms such as fever and pneumonia. Serious cases cause rapid death of patients. As of the end of February 2020, the COVID-19 epidemic has appeared in all continents except Antarctica, more than 80,000 people have been diagnosed worldwide, and the death toll has approached 3,000, seriously threatening global public health [1].

SARS-CoV-2 was first isolated from epithelial cells of human airway, and was identified by next-generation sequencing technology in January 2020 as a new member of β -CoVs coronavirus [2]. Coronaviruses are enveloped, single-stranded RNA viruses [2]. From 2002 to 2003, more than 8,000 patients were infected by the coronavirus which caused severe acute respiratory syndrome (SARS), and the WHO final reported 774 related deaths. Since September 2012, a total of 2494 laboratory-confirmed Middle East respiratory syndrome coronavirus (MERS-CoV) infections infected cases. The WHO has reported 858 virus-related deaths [3, 4]. All these emerging infectious diseases with global transmission are caused by β coronaviruses.

Although coronaviruses cause respiratory tract infections, several studies have shown that viral RNA of SARS-CoV [5], MERS-CoV [6], or SARS-CoV-2 [7, 8] can be detected in blood plasma. The WHO noted as early as 2003 that blood products could be contaminated with SARS-CoV virus. Although no SARS-CoV cases due to 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 found in COVID-19 cases [10], blood safety requires more attention [11, 12]. In addition, there are currently no specific drugs or vaccines effective against SARS-CoV-2. Therefore, patient convalescent plasma therapy should 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 plasma SARS-CoV-2 inactivation method.

Photochemical treatment methods are widely utilized pathogen inactivation/reduction technologies (PRTs). Photochemical methods for killing viruses are usually 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 often used clinically as an antidote to treat methemoglobinemia and cyanide poisoning caused by nitrite poisoning. Methylene blue surface carries a positive charge. It can be embedded in DNA or RNA, especially in combination with negatively charged G-C base pairs of viral nucleic acids. Under visible light, methylene blue absorbs light energy, activates 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 attack. Methylene blue can cause nucleic acid strand breaks under light [14]. The visible light source can be a halogen lamp, a metal halide lamp, or a fluorescent lamp, with full-band white light. However, due to the low excitation efficiency of methylene blue in full-band white light, this process also generates heat to destroy plasma components. The 'BX-1 AIDS treatment instrument' developed by Boxin (Beijing) Biotechnology Development LTD uses LED single wavelength light combined with methylene blue has higher effectiveness at killing various lipid enveloped viruses, such as HIV-1 and others [15, 16]. As SARS-CoV-2 is a new type of coronavirus, its tolerance to physicochemical conditions is unclear. Therefore, in this study, the 'BX-1 AIDS treatment instrument' was used to study the plasma inactivation of the SARS-CoV-2 isolate of Zhejiang University.

Results

Effect of SARS-CoV-2 infection on VERO cells

100 μ L of 4 lgTC ID₅₀/mL of SARS-CoV-2 was seeded into a 96-well plate with monolayers of VERO cells and cultured to observe the cytopathic effect (CPE). SARS-CoV-2 had a strong cytopathic effect, and the infected cells showed obvious pathological necrosis and large-scale detachment (Figure 1D).

Inactivation of BX-1 methylene blue photochemical method on SARS-CoV-2 virus

100 μ L of 4 lgTC ID₅₀/mL of SARS-CoV-2 was seeded into a 96-well plate with a single layer of VERO cells, and 1 μ M (Fig. 1A), 2 μ M (Fig. 1B), or 4 μ M of methylene blue (Fig. 1C) was added. Blue light was applied for 40 mins. The control groups were the non-lighted group (Figure 1E) and the methylene blue-only lighted group (Figure 1F). All treatments were cultured and observed for cytopathic effects (CPE). 1 μ M (Fig. 1A), 2 μ M (Figure 1B), and 4 μ M of methylene blue (Fig. 1C) can completely inactivate the virus, and the inactivated virus has no effect on cell growth. The non-illuminated group (Fig. 1E) and the methylene blue-only with light 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 3 passages of cell blind transmission, no cell lesions appeared in any generation of cells, indicating that the virus had been completely inactivated. qRT-PCR results indicated that virus plasma containing 1 μ M, 2 μ M, and 4 μ M of methylene blue can completely inactivate the virus in 2

minutes (Table 1).

Viral titer detection of the BX-1 methylene blue photochemical method for inactivation of SARS-CoV-2 virus

Using the CPE method, we found that the BX-1 methylene blue photochemical method can inactivate the SARS-CoV-2 virus in 40 mins. In order to further judge the inactivation efficiency, we established a 0, 2, 5, 10, 20, and 40 min time gradient. Viral titer tests found that virus plasma containing 1 μM and 2 μM methylene blue had a virus reduction $> 4\text{LgTCID}_{50}$ for 2 mins; the group with 4 μM methylene blue had an initial titer due to differences between batches in the test. For 3.5 LgTCID_{50} , the virus reduction was 3.5 LgTCID_{50} at 2 mins (Figure 2)

Discussion

The new coronavirus SARS-CoV-2, which appeared in December 2019, spread globally within three months and was 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 COVID-19 patients 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 for the use of pathogens to inactivate blood products (pathogen inactivation/reduction technologies (PRTs)) require exploration as soon as possible.

Coronaviruses are enveloped, single-stranded RNA viruses. Previous studies indicated that coronaviruses are generally susceptible to acid, alkaline, and heat [17]. After the outbreak of SARS and MERS, some studies investigated the potential of PRT to reduce or completely eliminate the potential risks of coronavirus transmission through blood products or derivatives [18]. 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 [19], and 25 mins at 56°C reduced MERS virus by more than 4 \log_{10} $\text{TCID}_{50}/\text{mL}$ [20]. Because heating can

denature proteins in blood products, it can only be used for manufacturing plasma-derived products;

(2) Photochemical treatment methods. Different wavelengths of light affect the viability of SARS and MERS viruses in the blood. Ultraviolet (UV) light, Amotosalen, or riboflavin can inactivate pathogenic nucleic acids [19]. Because the penetrating power of UV light is low, the inactivation efficiency is not high enough, especially when blood bags are used. It has been previously reported that methylene blue plus visible light is capable of inactivating coronavirus in plasma [18, 21] However, the wavelength range of visible light is large, which will increase the heating effect and cause plasma protein denaturation. 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 with 1 μ M methylene blue for 30 min. This can inactivate SARS-CoV and does not cause the loss of antibodies such as IgM and IgG in the plasma [22, 23]. Thus, its effect is far superior to the above methods. The new BX-1 'AIDS treatment instrument' used in this study can reduce the 4.5 log₁₀ TCID₅₀/mL in 2 mins for the new coronavirus SARS-CoV-2 (Figure 2), which is a strong advantage for inactivation of plasma viruses.

BX-1's highly efficient and safe SARS-CoV-2 plasma virus inactivation technology can not only be applied to the safety of blood transfusion, but also be used to directly treat the plasma of critical COVID-19 patients. Convalescent plasma therapy is a rapid and effective treatment 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 of animals. 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. Emil Berhring won the 1901 Nobel Prize in Medicine for his research on diphtheria serum therapy. The earliest convalescent plasma therapy in humans 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 [24]. Previously, in clinical treatment of SARS coronavirus, doctors in Hong Kong, Taiwan, and Singapore also tried convalescent plasma therapy for

critical SARS critical patients for whom comprehensive treatment had not been effective [25]. Given this long history of serum-based treatment in the modern era, and its established safety and efficacy, convalescent plasma transfusion should be used for COVID-19 patients [26]. Further, BX-1's effective virus inactivation technology can also be applied to the development of SARS-CoV-2 inactivated vaccines.

Materials And Methods

1. Cells, instruments, reagents, viruses

VERO-E6 cells are stored in the laboratory of Zhejiang University. DMEM medium, FBS, P/S, and PBS buffer are GIBCO products. Methylene blue is a product of Jichuan Pharmaceutical Group Co., Ltd. Human plasma is donated by volunteers. All experimental operations involving live viruses were carried out in Zhejiang University's Biosafety Tertiary Laboratory (BSL-3). The research protocol was approved by the Ethics Committee of the First Affiliated Hospital of Zhejiang University Medical College.

2. Methylene blue light inactivated virus

To 180 mL of healthy human plasma, 20 mL of indicator virus was added at a 9: 1 (V: V) ratio, and mixed. 1 methylene blue (MB) injection for clinical use (20mg / 2mL / branch) was diluted 13.4 times with normal saline to make up a 2mM / L methylene blue volume solution, and then diluted to 2000, 1000 and 500 times. This mixture was added to 200 mL of plasma virus mixture and mixed to a final concentration of 1 μ M, 2 μ M, or 4 μ M. "BX-1 AIDS treatment instrument" was used at room temperature, illumination adjusted to $55,000 \pm 0.5$ million Lux, and irradiated 0, 2, 5, 10, 20, 40 mins under single wavelength of 630nm light. About 1 mL of plasma was taken at each time point, diluted 10-fold with DMEM medium containing 2% FBS, and then filtered through a 0.45 μ m filter for virus titer detection. At the same time, the virus control was set (only virus in the plasma, left at room temperature for 40 mins as an untreated control), and control with pure methylene blue (MB) was set (virus and methylene blue 4 μ M added to the plasma, with no light treatment, and allowed to stand for 40 mins as a control for the effect of methylene blue on the virus) and a finally the 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 virus).

3. Virus titer measurement

After trypsinization of VERO cells, 1×10^4 cells/well were inoculated into 96-well cell culture plates at 100ul of culture medium per well. After the cells grew into a single layer in a 96-well plate, the culture medium was discarded and the treated plasma was seeded. The virus was log-diluted with 2% FBS in DMEM medium from 10⁻²-10⁻⁷. This process was repeated for 4 wells per dilution, 200ul/well. Normal cell control wells were established (with cells, virus-free). The 96-well plate was placed in a 5% CO₂ and 37 ° C incubator. After 3 h of incubation, the supernatant was washed off, and 200 uL/well of 2% FBS DMEM medium was added. Cell lesions were observed every 24 h until 6 d. TCID₅₀ 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.

4. Three Generations of Blind Tests

1 mL of the test group with virus and 1,2,4 uM methylene blue was irradiated for 40 min. It was then diluted 10 times with 2% FBS DMEM medium, filtered with 0.45 µm filter (Millipore), and added to VERO cells every 48 h. 1 mL of the supernatant was diluted 10 times with 2% FBS DMEM medium, and added to VERO cells for 3 passages. These were observed for cytopathic effects. For the duration of the 3rd generation of cell blind transmission, cytopathic lesions were positive (+) at any time, indicating that the virus was not completely inactivated; negative (-), indicating that the virus had been completely inactivated.

5. Viral load of culture supernatant measured by qRT-PCR

Nucleic acid extraction: with 200uL of virus culture supernatant, the MVR01 magnetic bead method nucleic acid extraction kit was used (article number: ZM-0044, Shanghai Zhijiang Biotechnology Co., Ltd.) in the EX2400 automatic nucleic acid extraction instrument (Shanghai Zhijiang Biotechnology Co., Ltd. Virus nucleic acid was extracted, and the elution volume was about 50 µl.

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 viral load. The specific steps were as follows: $n \times 19$ uL of new coronavirus (2019-nCoV) nucleic acid fluorescent PCR detection mixture and $n \times 1$ uL RT-PCR enzyme (n is the number of reaction tubes) were shaken and mixed for several seconds, and centrifuged at 3000 rpm for several seconds. 20 uL of the above mixture was placed into a PCR

tube, to which was added 5 uL each of the sample nucleic acid extraction solution, DEPC-H₂O, and positive control to the PCR tube. The tube cap was covered and the PCR amplification reaction was immediately performed. The PCR amplification reaction tube was placed on a LightCycler® 480II (Roche) real-time quantitative PCR instrument, and FAM and VIC (or TEXAS RED) fluorescence channels were selected for detection. Recommended cycle parameter settings: 45 °C × 10min; 95 °C × 15min; then 95 °C × 15sec, 60 °C × 60sec, cycle 45 times; single-point fluorescence detection at 60 °C. A CT value below 35 was considered effective amplification, and a CT value above 35 was considered undetected.

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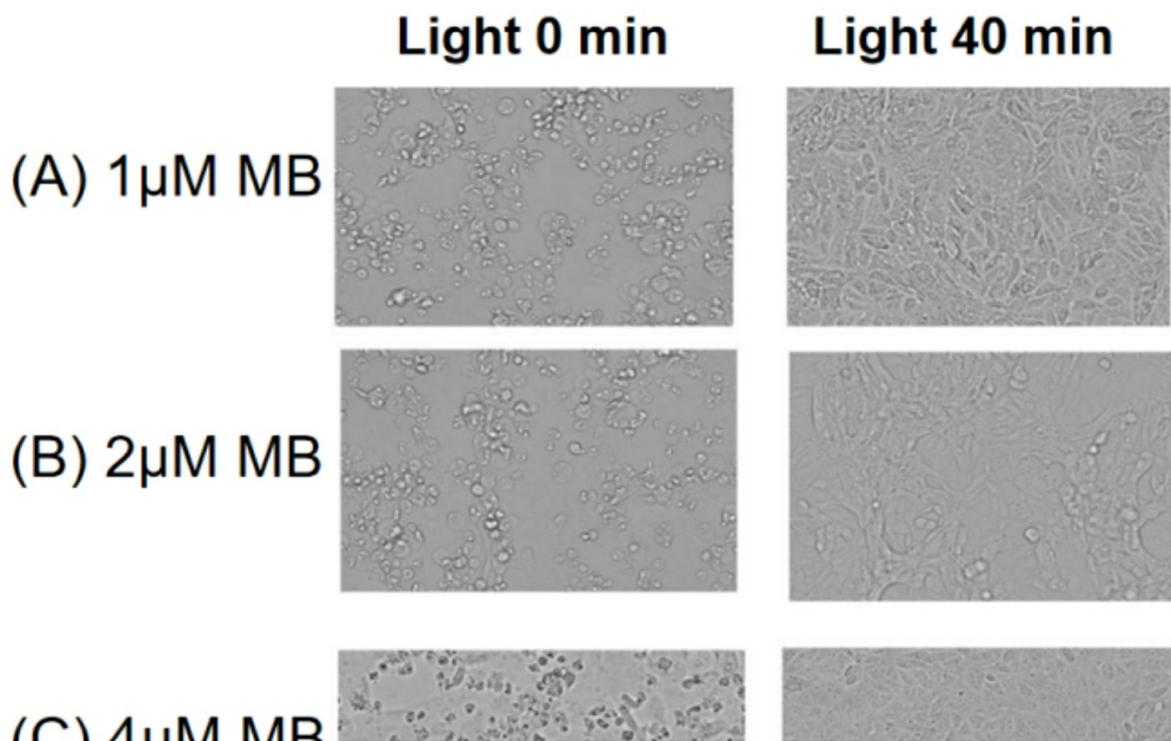
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Table

Due to technical limitations, table 1 is only available as a download in the supplemental files section.

Table 1 Plasma SARS-CoV-2 virus titration test, with CT dilution of supernatant and nucleic acid qRT-PCR detection. Note: '/' indicates not applied. ND indicates not detected

Figures



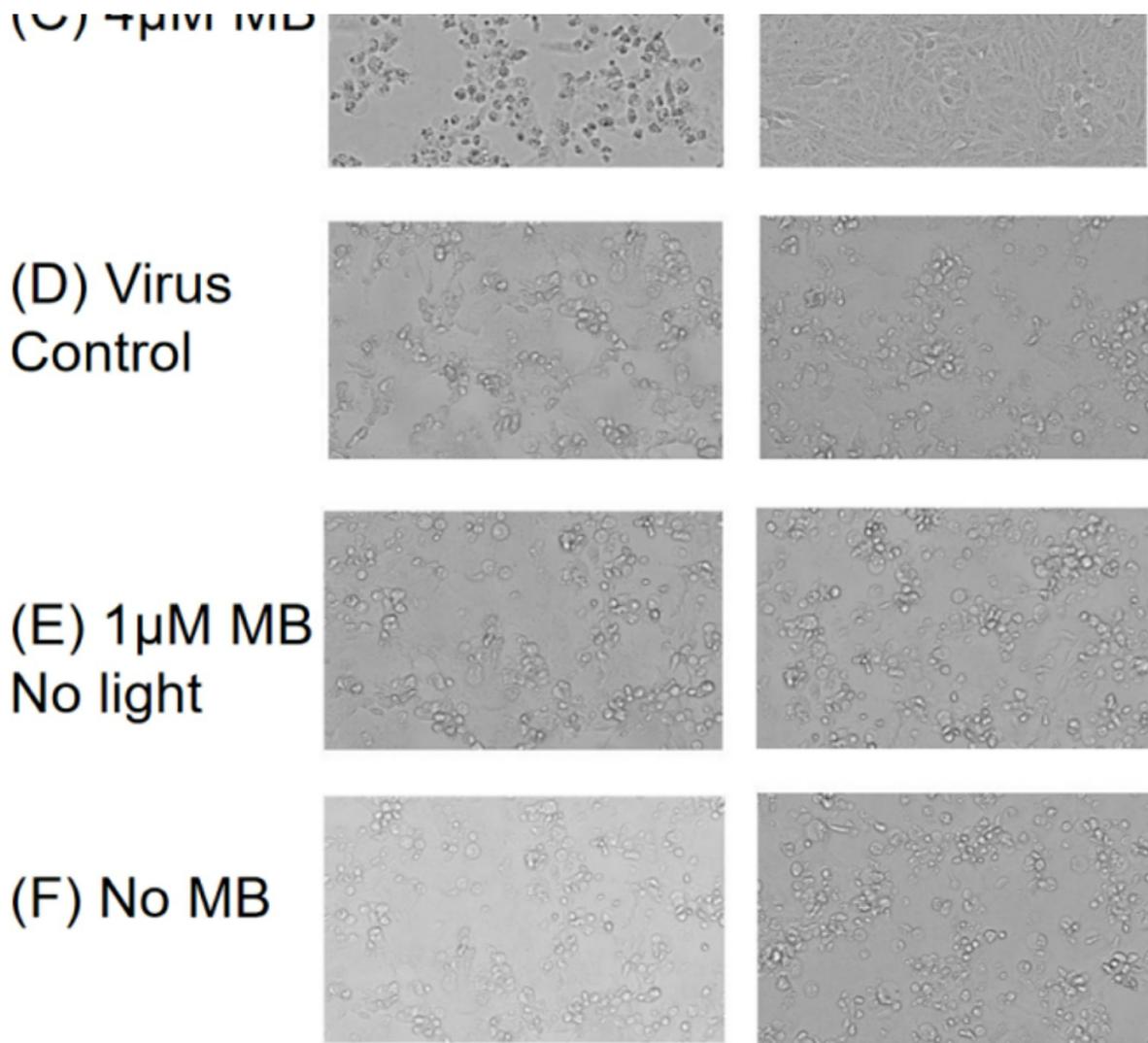


Figure 1

VERO cells infected by SARS-CoV-2 virus across different treatment groups (100X). 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 μ M (A), 2 μ M (B), or 4(C) μ M. "BX-1 AIDS treatment instrument" was used at room temperature, illumination adjusted to $55,000 \pm 0.5$ million Lux, and irradiated 40 mins under single wavelength of 630nm 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 μ M MB (E) was set (virus and methylene blue 1 μ M added to the plasma, with no light treatment, and allowed to stand for 40 mins as a control for the effect of methylene blue on the virus) and a 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).

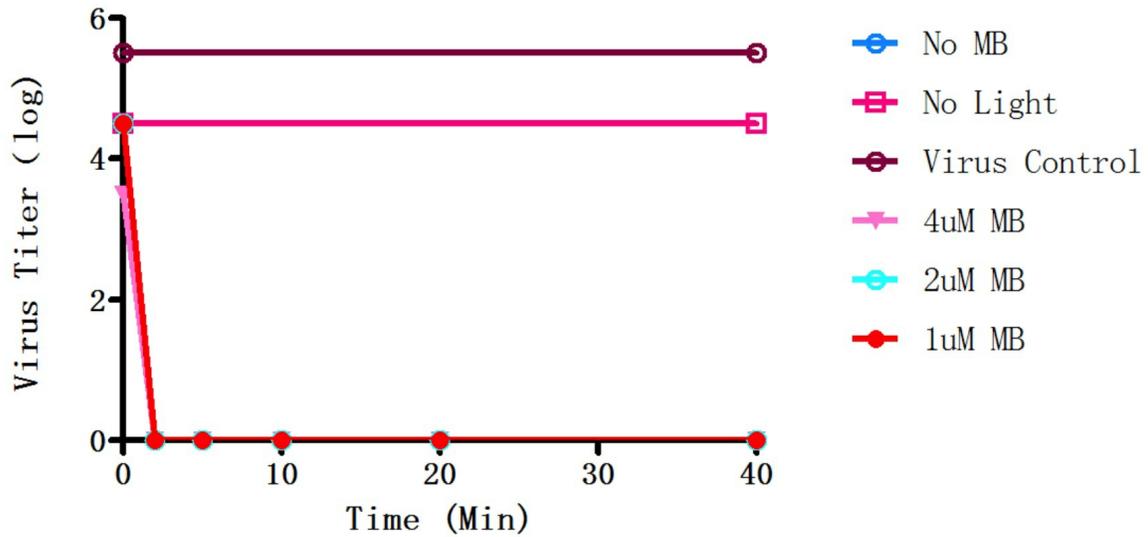


Figure 2

'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 μ M methylene blue group and the 2 μ M methylene blue group. This experiments were repeated three time respectively ($p < 0.01$).

Supplementary Files

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Table 1.jpg