



Rational Use of Methylene Blue in COVID-19 Treatment

Mr. Saikrupa BV^{1*}, Dr. Muthukumar Mani², Dr. Kavya S³, Dr. Suma P Kumar⁴

^{1,2} Department of Pharmacy Practice, Faculty of Pharmaceutical sciences, PES University, Bangalore [formerly PES College of Pharmacy, Bangalore].

^{2,4} Excel care hospital, banashankri stage 2, Bangalore.

Article Info:



Article History:

Received 13 March 2022

Reviewed 16 April 2022

Accepted 19 April 2022

Published 15 May 2022

Cite this article as:

Saikrupa BV, Muthukumar M, Kavya S, Suma PK, Rational Use of Methylene Blue in COVID-19 Treatment, Journal of Drug Delivery and Therapeutics. 2022; 12(3):181-186

DOI: <http://dx.doi.org/10.22270/jddt.v12i3.5438>

*Address for Correspondence:

Saikrupa B V, Pharm. D [Doctor of Pharmacy], Department of Pharmacy Practice, Faculty of Pharmaceutical sciences, PES University, Bangalore-560050 [formerly PES College of Pharmacy, Bangalore].
ORCID ID: <https://orcid.org/0000-0002-3770-8358>

Abstract

Methylene blue entered the medical field when Robert Koch and Paul Ehrlich introduced the staining of tuberculosis microorganisms using methylene blue. It is used as an anesthetic agent for its sensory nerve ending blocking property, and its antimicrobial activity was useful as an antimalarial therapy in the 1890s. In the modern medical field, methylene blue was majorly used for investigational purposes as contrast in many radiological diagnostic techniques. Further applications of methylene blue in various clinical indications like Alzheimer's disease, depression, and psychosis are under investigation. Human Coronavirus invasion through the ACE 2 receptor causes the immune system's activation and induces apoptosis in human cells like monocytes, macrophages, T lymphocytes, and dendritic cells, which results in the activation of the innate and acquired immune system. This process results in the massive elimination of the infected cells. Methylene blue can play a significant role in treating SARS-CoV-2 infection by inhibiting viral replication and preventing sepsis due to covid illness. Theoretically, anti-inflammatory activity, oxidative stress, and tissue damage caused by covid infection can be minimized, and thereby it can also be beneficial for the patients suffering from cytokine storm. From this review article, we conclude that methylene blue should be evaluated for its role in covid treatment at a clinical level.

Keywords: SARS-CoV-2, Antiviral activity, Cytokine storm, Nitric oxide, Antidote activity.

1. INTRODUCTION

In November 2020 FDA approved a clinical trial for using Methylene blue (MB) in covid treatment, and now the participants are in phase 2 trial¹. In 1876 methylthioninechloride(methylene blue) was synthesized by a German chemist, Heinrich Caro, which is used as an aniline-based dye for cotton dying industrial usage. Various applications within the medical field have been explored, and currently, there are >21000 entries available in PubMed for "methylene blue"^{2,3}.

Methylene blue (MB) entered the medical field when Robert Koch and Paul Ehrlich introduced the staining of tuberculosis microorganisms using MB⁴. MB has been used as an anesthetic since the 1890s, and currently, its sensory nerve ending blocking property has been used for local anesthesia in higher concentration⁵. Within decades the MB entered into the realm of the laboratory. With the invention of its antimalarial activity by Ehrlich (1891), the primary chemical moiety was employed as an antimicrobial agent⁶.

Modern biomedical fields have widened the usage of MB in cancer diagnosis as well as therapeutics. The essential use of MB is found to be in the treatment of methemoglobinemia because of exposure to certain chemicals like nitrophenols, poisons like cyanide, etc⁷. Further applications of MB in various clinical indications like Alzheimer's disease, depression, and psychosis are under evaluation^{8, 9,10}.

2. PATHOLOGY OF SARS-CoV-2

The first reported case of SARS COV 2 was found in the city of Wuhan, Hubei province republic of china, in December 2019. The genetic sequence of the novel coronavirus was studied and released in mid of January 2020¹¹. Coronavirus has trimeric spike-like structured (S) glycoprotein on its surface. This S glycoprotein initiates the binding of the virus onto the host cell surface protein angiotensin-converting enzyme 2, and this S protein is composed of subunits such as S1, which helps in the receptor binding, and S2 in the fusion of the virus envelope with the human cell membrane and release the nucleocapsid into the cytoplasm¹².

When the viral nucleocapsid enters into the cytoplasm, the virus's replication process starts with the translation of ORF 1a and 1b into polyproteins pp1a and pp1ab. Replicated new genomic RNA will be molded with helical nucleocapsid, which interacts with viral structural proteins¹³. Replicated virions are transported through vesicles and released from the cell by the process of exocytosis. In this process, many proteins play a significant role, such as E protein and M protein^{14,15}.

Human Coronavirus invasion will cause activation of the immune system and induce apoptosis in human cells such as monocytes, macrophages, T lymphocytes, and dendritic cells that result in the activation of the innate and acquired immune system. This process results in the massive elimination of the infected cells¹⁶ Studies reveal that the coronavirus damages

dendritic cells, enhancing the spread of the virus and activating the host immune system¹⁷.

Kallikrein-Kinin System: This system consists of many proteins and peptides and possesses major physiological roles in the heart, blood vessels, skin hemostasis, ocular functions, coagulation cascade, inflammation, and pain pathway¹⁸. SARS COV 2 enters into the lungs and interacts with ACE2 (Angiotensinogen converting enzyme) receptors and downregulates its function and this leads to activation of bradykinin receptor B1 and also increases pro-inflammatory mediators release such as TNF (Tumor necrosis factor), MIP2 (Macrophage inflammatory protein). This interaction also causes neutrophil infiltration and exaggerates inflammatory processes, and can result in angioedema in infected patients^{19,20}.

This downregulation of ACE activity causes an imbalance in the RAAS [Renin angiotensin aldosterone system] pathway, which leads to an increase in cytokine synthesis by activation of Nitric oxide (NO) and prostaglandins synthesis; this causes exudation and vascular leakage and stimulate the formation of free radical and inhibit the production of ATP in mitochondria by altering TCA cycle and the glycolytic pathway. The appearance of pro-inflammatory cytokines such as IL-17, IL-6, IL-2, IL-1B, IL-8, TNF-alpha, and IL-1beta leads to stimulation of NO synthesis²¹. IL-2 is massively high in covid-19 subjects²², and IL-2 is known to stimulate NO formation in covid-19 subjects²³. Nitric oxide acts as a key mediator of IL-2 and causes vascular leak syndrome and hypotension²⁴. IL-6 up-regulates inflammatory cytokines in covid-19²², TNF-alpha and IL-6 stimulate superoxide radicals in neutrophils^{25,26}, and hydrogen peroxide can promote the formation of IL-6 and other inflammatory mediators²⁷. This leads to the formation of cytotoxicity in the cell and further leads to blockage of lipid peroxidation²⁸ and apoptosis. This is a life-threatening condition in covid-19 patients called cytokine storm²⁹. Another important impact of kallikrein-kinin system dysregulation is an imbalance in coagulation cascade which tends to increase the formation of microthrombi³⁰, which is well known to occur in COVID19 patients.

3. THE PHARMACOLOGICAL ACTIVITY OF METHYLENE BLUE THAT CAN SUPPORT IT AS A THERAPEUTIC AGENT IN SARS-COV-2

3.1. Antiviral activity:

Methylene blue has a virucidal effect. It acts by interacting between the coronavirus spike protein (macromolecules) and the ACE2 receptor.³¹ It prevents the maturation of phagosomes and also inhibits replication and translation of virus through interaction with RNA. It prevents substrate-level phosphorylation in the mitochondria and fosters the formation of hydrogen peroxide (H₂O₂) ions, thereby leading to oxidative destruction of the SARS-CoV2. It alters intracellular pH by alkalization of endosomes and lysosomes, which help to decontaminate the virus. Methylene blue and its reduced and unchanged derivative (leukoMB) can easily cross the membrane, causing an increase in intracellular pH and endosome maturation. Cells are blocked at intermediate stages due to an increase in intracellular pH, further penetrating virions into the cytoplasm.^{32,33}

Experimental studies of Methylene blue in inactivation of virus in convalescent plasma therapy is explained by the following; The viral RNA of SARS CoV2^{34,35} or MERS³⁶ (Middle East Respiratory Syndrome), SARS-CoV³⁷, is detected in blood plasma. WHO predicted in early 2003 that SARS-CoV2 could contaminate blood products. So far, none of the cases have been reported due to blood transfusion, but theoretically, there could still be the possibility of a blood transfusion-

related infection³⁸. But in this critical scenario, the safety of plasma/blood transfusion is more important^{39,40}. Convalescent plasma therapy could be an effective and rapid treatment for SARS-CoV2^{41,42}. However, in this situation, it is necessary to inactivate the viral pathogen in the plasma. This can be done by photochemical treatment, which is widely used for inactivating viral and bacterial pathogens. This is done by adding methylene blue to blood products in the presence of ultraviolet light using a photochemical process. Thiazine ring of methylene blue undergoes a redox reaction to collect electrons, which are then converted to leuco-methylene blue, and then electrons are transferred to other components. It also combines with oxygen, and an energy source like metal halide lamp, halogen lamp, or fluorescent⁴³ lamp enters into electrochemical ground state reactions in the singlet-oxygen state. This often forms a highly reactive analog, oxidation of guanine (8oxo7, 8-dihydroguanine (8oxoGua))^{44,45} that damages the viral DNA or RNA of the coronavirus.^{46,47,48}

3.2. Anti-inflammatory activity:

Cytokine release syndrome seen in the severe covid19 patients will exhibit a considerable increase in pro-inflammatory cytokines such as IL-17, IL-6, IL-1B, IL-8, IL-2, and TNF-alpha. This is all mediated by NOD (Nucleotide-binding oligomerization domain)-like receptor protein 3 (NLRP3), which regulates inflammation. This results in tissue damage and organ failure. Methylene blue inhibits the formation of the NLRP3 protein complex, thereby reversing the mRNA expression and alter the stimulation of cytokines like IL-12, IL-10, IL-6, IL-1alpha^{49,50}. Methylene blue also alters cytokine production via NF-kb (Nuclear factor kappa light chain enhancer of activated B cells) signaling pathway^{51,52} and is hence useful as a therapeutic option.

3.3. Rejuvenation of Mitochondria and Superoxide inhibition:

1. Normally, cell cellular respiration takes by the process of glycolysis in cytoplasm and Krebs cycle in mitochondria. This is by the process of oxidative phosphorylation. NAD⁺ is the coenzyme used in cellular respiration to transport high potential energy electrons to the electron transport chain in the mitochondria. This leads to the production of energy in the form of ATP. In hypoxia conditions, the formation of NAD⁺ is inhibited. Hence, pyruvate, which is the product of glycolysis, reduces lactate dehydrogenase. This is an anaerobic glycolytic pathway that reduces the pH in the cell, causing acidosis. MB administered gets converted to leuco-methylene blue, which helps in the restoration of NAD⁺ and hence glycolysis. Methylene blue also acts as a coenzyme in the electron transport chain. It also promotes formation of acetyl CoA from pyruvate rather than lactic acid⁴⁹.

2. Due to imbalance within the mitochondria of infected host cells, there is formation of free radicals such as superoxides, hydrogen peroxide, peroxy radicals, hydroxyl radical⁵³. Free radicals are formed during intracellular redox reactions in mitochondria and are essential for many physiological functions such as gene expression, signal transduction, and massive amounts in Covid19 of free radicals, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are formed^{21,54}. This leads to the inhibition of endogenous proteins, nucleic acids, lipids, ROS can significantly regulate the cell proliferation adaptations and irreversible damage to the cell⁵⁵. ROS formation is inhibited by Methylene blue by blocking the xanthine oxidase enzyme^{21,56}. And hence useful in therapeutic options.

3.4. Nitric oxide synthase enzyme inhibition:

Nitric oxide(NO) acts as a signaling molecule between endothelial and smooth muscle through the conversion from

L-arginine to nitric oxide, which is mediated by Nitric oxide synthase. It is mediated by endothelial-derived relaxing factor (EDRF), as a response to stimulation of certain hormones and neurotransmitters leading to stimulation of vascular endothelial cells⁵⁷. It causes the influx of Ca²⁺ ions, which leads to activation of calmodulin complex, which helps in the conversion of an inactivated form of NO to the activated form of NO⁵⁸. Methylene blue blocks the guanylate cyclase enzyme, which is responsible for retraction of free heme group and smooth muscle contraction^{59,61}. Methylene blue blocks nitric oxide synthase enzyme⁶¹. Inhibition of NO synthesis can lead to decrease in IL-6 formation more than 50%⁶² and hence useful as a therapeutic option.

3.5. Antidote activity:

According to case reports of Covid-19, in some patients hypoxia is due to the formation of met-hemoglobin in the blood.^{63,64}. Hemoglobin is formed by the combination of iron (Fe³⁺) and globin. Met-hemoglobin is a type of hemoglobin formed spontaneously in the human blood and is bluish chocolate brown in color. Met hemoglobin doesn't carry oxygen to the tissues, and it is reduced to hemoglobin by the NADH or NADPH-dependent methemoglobin reductase enzyme. Ingestion of certain pharmaceutical chemicals, toxins, or broad beans leads to methemoglobinemia. The higher concentration of methemoglobin in the blood decreases the oxygen supply to the cells/tissues. MB acts as an antidote for

methemoglobin. When MB is administered intravenously, it converts itself into leucomethylene blue reducing heme content from methemoglobin, converting it to hemoglobin which increases oxygen delivery to the tissues⁶⁵.

4. PHARMACOKINETICS.

The details about the Absorption of methylene blue are poorly understood. The volume of distribution of methylene blue is found to be 10 mg/kg in the studies carried out on rats. MB is a highly protein binding drug (71–77%). It is readily distributed in muscle tissue. Up to 75% of MB is excreted through urine as leucomethyleneblue. Significant amount of MB was found in bile. IV administration MB has shown 5–6.5hrs of half-life⁶⁶.

5. CONTRAINDICATION

Methylene blue use is contraindicated in people who have developed hypersensitivity reaction to it, Glucose-6-phosphate dehydrogenase (G6PD) deficiency as it can cause severe hemolysis^{67,68}. Its use is contraindicated in pregnancy (Category X) as it can cause fetal death⁶⁹. MB should not be combined with Antidepressants such as Selective serotonin reuptake inhibitors [SSRIs], Selective Non-serotonin reuptake inhibitors SNRIs, Mono-amino-oxidase inhibitors [MAOIs], and Tricyclic antidepressants [TCAs] can cause serotonin release syndrome⁷⁰ which can be life threatening.

6. DOSE OF METHYLENE BLUE

Table.no 1

Route Of Administration	Treatment Regimen
a) Intravenous(Iv)	Initially, give high dose Vit B complex and 2 tab of Pyridium 2 hours before MB. Followed by 5gm Vit C with 100mg MB in 200ml N saline for 4hrs before IV infusion. Or 1mg of MB in 100ml saline over 1 hour for 3days*. 1mg/kg body weight in ICU admitted patients with highly oxygen requirement (SpO ₂ <200) ⁷¹
b) Nebulizer	5ml of 0.1% MB + 1ml Dexamethasone sodium + 1 ml of levosalbutamol and ipratropium bromide respirator solution*.
C) Oral	2-3mg/kg/day orally in 3 divided doses for 7 to 10 days (For newly diagnosed (initial) covid-19 patients) ⁵⁴

(*As per webinar proceedings – Dr.Golwalker's protocol; <https://youtu.be/CS1CxuAfd-U>)

7. ADVERSE DRUG REACTIONS ASSOCIATED WITH THE METHYLENE BLUE:

Urine discoloration is frequently reported ADRs of MB. IV administration of MB can cause limb pain. Methylene blue can cause CNS-related side effects such as dizziness, confusion, and headache⁷². Administration of methylene blue can cause hemolytic anemia, pulmonary edema, respiratory depression, hyperbilirubinemia, and phototoxicity, which is noticed in neonates^{73,74}. Hence patients should be closely monitored on this.

CONCLUSIONS:

The covid-19 disease predominantly damages lung and vascular endothelium because of the presence of ACE2 receptors, which provide the entry point. This results in the production of reactive oxygen species and excess cytokine release that causes tissue damage. The virus enters into the interstitial space, causing oxidative stress and results in apoptosis or necrosis and damage to vascular endothelial tissue and alveoli. These cytokines, ROS and RNS, enter into the systemic circulation resulting in damage of endothelium of

other organs. The viral protein circulates and can lead to multi-organ failure. In covid-19, when ROS, RNS, and cytokine production are out of control and a single cytokine inhibitor can fail to show an effect.

According to current scientific research at this juncture, MB can act as a multi-modal therapy during covid-19 disease. From the above evidence, methylene blue can play a significant role in treating SARS-CoV-2 infection by inhibiting viral replication and reducing sepsis due to covid illness. By its anti-inflammatory activity, oxidative stress and tissue damage caused by covid infection can be minimized, and thereby it can also be used for covid-19 patients with mild to severe disease and also patients suffering from cytokine storms. Rational use of methylthioninium chloride (methylene blue) in SARS-CoV2 infection potentially reduces the mortality and morbidity rate. Therefore, this review article concludes that methylene blue can play a crucial role in covid treatment in all stages.

Abbreviations:

MB: Methylene blue; SARS-CoV-2: Severe acute respiratory distress syndrome-coronavirus-2; TNF: Tumor necrosis factor;

MIP2: Macrophage inflammatory protein; ACE: Angiotensin-converting enzyme; NO: Nitric oxide. RAAS = Renin angiotensin aldosterone system; PH: Potential of hydrogen; NOD: Nucleotide-binding oligomerization domain; ROS: Reactive oxygen species; RNS: Reactive nitrogen species; EDRF: Endothelial-derived relaxing factor; NF-kB: Nuclear factor kappa light chain enhancer of activated B cells; G6PD: Glucose-6-phosphate dehydrogenase; SSRIs: Selective serotonin reuptake inhibitors; SNRIs: Selective Non-serotonin reuptake inhibitors; MAOIs: mono-amino-oxidase inhibitors; TCA: Tricyclic antidepressants.

Acknowledgments:

I would like to extend my gratitude to Prof Dr. SRINIVASAN R (chairperson, department of pharmacy practice, PES University, Bangalore) and Prof Dr. SARAVANAN J (Dean of faculty of pharmaceutical sciences, PES University, Bangalore.) for helping the team land this review topic, for permitting to utilize the resources sheltered in the womb of the college, motivating the team morally and with professional wisdom throughout the journey of this review work.

Author Contributions:

PES institution and Excel care hospital provided essential resources. Saikrupa B V and Dr. Muthu Kumar Mani are collected data and drafted the original script. Dr. Kavya S assisted with data collection and editorial works. Dr. SUMA P Kumar supervised the editorial works. All the authors read and approved the final manuscript.

Funding:

Not applicable

Availability of data and material:

Not applicable

Declarations

Ethics approval and consent to participate: Not applicable

Competing interests:

The authors have no conflicts of interest regarding this article.

REFERENCES

1. Clinical trial.gov[internet].USA: u.s. national library of medicine[updated may 4, 2020] Available Form:<https://clinicaltrials.gov/ct2/show/NCT04635605>
2. Pub med[internet]. Maryland: u.s. national library of medicine Available Form:<https://pubmed.ncbi.nlm.nih.gov/?term=methylene+blue>
3. Alhenc-Gelas F, Girolam's JP. Molecular and genetic aspects of the Kallikerin-Kinin System. In: Michael Bader, editors. Kinins. German: De Gruyter, 2011, page no 7-37. <https://doi.org/10.1515/9783110252354.7>
4. Michael Titford, HTL (ASCP)HT. Paul Ehrlich: Histological Staining, Immunology, Chemotherapy. Laboratory Medicine, Volume 41, Issue 8, August 2010, Pages 497-498 <https://doi.org/10.1309/LMHJS86N5ICBIBWM>
5. Tan KY, Seow-Choen F. Methylene blue injection reduces pain after lateral anal sphincterotomy. Tech Coloproctol. 2007; 11:68-69 doi.org/10.1111/j.1445-2197.2007.04116.16.x
6. Guttmann P, Ehrlich P. Über die Wirkung des Methylenblau bei Malaria. In: The Collected Papers of Paul Ehrlich. Elsevier; 1960. p. 9-14. <https://doi.org/10.1016/B978-0-08-009056-6.50006-3>
7. Wainwright M, Crossley KB. Methylene Blue--a therapeutic dye for all seasons? J Chemother. 2002 Oct; 14(5):431 <https://doi.org/10.1179/joc.2002.14.5.431>
8. Wischik CM, Edwards PC, Lai RY, Roth M, Harrington CR. Selective inhibition of Alzheimer disease-like tau aggregation by phenothiazines. Proc Natl Acad Sci U S A. 1996; 93:11213-11218 <https://doi.org/10.1073/pnas.93.20.11213>
9. Naylor GJ, Martin B, Hopwood SE, Watson Y. A two-year double-blind crossover trial of the prophylactic effect of methylene blue in manic-depressive psychosis. Biol Psychiatry. 1986; 21:915-920 [https://doi.org/10.1016/0006-3223\(86\)90265-9](https://doi.org/10.1016/0006-3223(86)90265-9)
10. Narsapur SL, Naylor GJ. Methylene blue. A possible treatment for manic depressive psychosis. J Affect Disord. 1983; 5:155-1561 [https://doi.org/10.1016/0165-0327\(83\)90008-3](https://doi.org/10.1016/0165-0327(83)90008-3)
11. World Health Organisation. CoronaVirus Disease 2019 Situation report 94, 23 april2020[internet]. Geneva Available Form: <https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200423-sitrep-94-covid-19.pdf>
12. Masters PS. The molecular biology of coronaviruses. Adv. Virus Res. 2006; 66:193-292 [https://doi.org/10.1016/S0065-3527\(06\)66005-3](https://doi.org/10.1016/S0065-3527(06)66005-3)
13. Luo H, Chen Q, Chen J, Chen K, Shen X, Jiang H. The nucleocapsid protein of SARS coronavirus has a high binding affinity to the human cellular heterogeneous nuclear ribonucleoprotein A1. FEBS Lett. 2005 May 9; 579(12):2623-8 <https://doi.org/10.1016/j.febslet.2005.03.080>
14. Neuman BW, Kiss G, Kunding AH, Bhella D, Baksh MF, Connolly S, Droese B, Klaus JP, Makino S, Sawicki SG, Siddell SG, Stamou DG, Wilson IA, Kuhn P, Buchmeier MJ. A structural analysis of M protein in coronavirus assembly and morphology. J Struct Biol. 2011 Apr; 174(1):11-22 <https://doi.org/10.1016/j.jsb.2010.11.021>
15. Luo H, Wu D, Shen C, Chen K, Shen X, Jiang H. Severe acute respiratory syndrome coronavirus membrane protein interacts with nucleocapsid protein mostly through their carboxyl termini by electrostatic attraction. Int J Biochem Cell Biol. 2006; 38(4):589-99 <https://doi.org/10.1016/j.biocel.2005.10.022>
16. Gu J, Korteweg C. Pathology and pathogenesis of severe acute respiratory syndrome. Am J Pathol. 2007 Apr; 170(4):1136-47 <https://doi.org/10.2353/ajpath.2007.061088>
17. Spiegel M, Schneider K, Weber F, Weidmann M, Hufert FT. Interaction of severe acute respiratory syndrome-associated coronavirus with dendritic cells. J Gen Virol. 2006 Jul; 87(Pt 7):1953-1960 <https://doi.org/10.1099/vir.0.81624-0>
18. Michael Bader. Kinins. Walter de Gruyter GmbH & Co. KG, Berlin/Boston
19. Sodhi, C. P, et al. Attenuation of pulmonary ACE2 activity impairs inactivation of des-Arg9 bradykinin/BKB1R axis and facilitates LPS-induced neutrophil infiltration. American Journal of Physiology-Lung Cellular and Molecular Physiology, 314(1), L17-L31. <https://doi.org/10.1152/ajplung.00498.2016>
20. Shahad A. Abasaheed Elhag, MMBS, Halah Ibrahim, MD, MEHP, and Shaden Abdelhadi, MD. Angioedema and urticaria in a COVID-19 patient: A case report and review of the literature. JAAD Case Rep. 2020 Oct; 6(10):1091-1094. <https://doi.org/10.1016/j.jidcr.2020.07.042>
21. Jun wu, Nitric Oxide: Trackle the free radicals damage in covid-19, Elsevier, 2020; 102(1):39-41. <https://doi.org/10.1016/j.niox.2020.06.002>
22. Li Y, Hu Y, Yu J, Ma T. Retrospective analysis of laboratory testing in 54 patients with severe- or critical-type 2019 novel coronavirus pneumonia. Lab Invest. 2020; 100(6):794-800. <https://doi.org/10.1038/s41374-020-0431-6>
23. Hibbs, J.B., Jr., et al. Evidence for cytokine-inducible nitric oxide synthesis from Larginine in and vascular leak syndrome. J Immunother patients receiving interleukin-2 therapy. J Clin Invest, 1992; 89(3):867-77. <https://doi.org/10.1172/JCI115666>
24. Samlowski, W.E., et al. Endothelial nitric oxide synthase is a key mediator of interleukin-2-induced hypotension, 2011; 34(5):419-27. <https://doi.org/10.1097/CJ1.0b013e31821dcb50>

25. Tsujimoto, M., et al., Tumor necrosis factor provokes superoxide anion generation from neutrophils. *BiochemBiophys Res Commun*, 1986; 137(3):1094-100. [https://doi.org/10.1016/0006-291X\(86\)90337-2](https://doi.org/10.1016/0006-291X(86)90337-2)
26. Kharazmi, A., et al., Interleukin 6 primes human neutrophil and monocyte oxidative burst response. *Immunol Lett*, 1989; 21(2):177-84. [https://doi.org/10.1016/0165-2478\(89\)90056-4](https://doi.org/10.1016/0165-2478(89)90056-4)
27. Colston, J.T., B. Chandrasekar, and G.L. Freeman, A novel peroxide-induced calcium transient regulates interleukin-6 expression in cardiac-derived fibroblasts. *J Biol Chem*, 2002; 277(26):23477-83. <https://doi.org/10.1074/jbc.M108676200>
28. Rabinovitch, A., et al., Cytotoxic effects of cytokines on rat islets: evidence for involvement of free radicals and lipid peroxidation. *Diabetologia*, 1992; 35(5):409-13. <https://doi.org/10.1007/BF02342435>
29. Casey LC. Role of cytokines in the pathogenesis of cardiopulmonary-induced multisystem organ failure. *Ann ThoracSurg* 1993 Nov; 56(5 Suppl):S92-6. [https://doi.org/10.1016/0003-4975\(93\)91143-B](https://doi.org/10.1016/0003-4975(93)91143-B)
30. Patricia Ribeiro de Carvalho,aPierre Sirois,b and Patricia Dias Fernandes,* The role of kallikrein-kinin and renin-angiotensin systems in COVID-19 infection. *Peptides*. 2021 Jan; 135:170428. <https://doi.org/10.1016/j.peptides.2020.170428>
31. Bojadzic D, Alcazar O, Buchwald P. Methylene Blue Inhibits the SARS-CoV-2 Spike-ACE2 Protein-Protein Interaction-a Mechanism that can Contribute to its Antiviral Activity Against COVID-19. *Front Pharmacol*. 2020; 11:600372. <https://doi.org/10.3389/fphar.2020.600372>
32. Crooks J,Haemolytic jaundice in a neonate after intra-amniotic injection of methylene. *Archives of disease in childhood*. 1982 Nov; <https://doi.org/10.1136/adc.57.11.872>
33. Cagno V, Medaglia C, Cerny A, Cerny T, Zwygart AC-A, Cerny E, et al. Methylene Blue has a potent antiviral activity against SARS-CoV-2 and H1N1 influenza virus in the absence of UV-activation in vitro. *Sci Rep*. 2021; 11(1):14295. <https://doi.org/10.1038/s41598-021-92481-9>
34. Huang C, W.Y., Li X, Ren L, Zhao J, Hu Y., Clinical features of patients infected with 2019 novel coronavirus in Wuhan *Lancet*, 2020 [https://doi.org/10.1016/S0140-6736\(20\)30183-5](https://doi.org/10.1016/S0140-6736(20)30183-5)
35. Kim JY, K.J., et al., Viral Load Kinetics of SARS-CoV-2 Infection in First Two Patients in Korea. *J Korean Med Sci*, 2020; 35(7):e86. <https://doi.org/10.3346/jkms.2020.35.e86>
36. Corman VM, A.A., Omrani AS, Albarrak MM, Farah ME, Almasri M. Viral shedding and antibody response in 37 patients with Middle East respiratory syndrome coronavirus infection. *Clin Infect Dis*, 2016; 62:477-83 <https://doi.org/10.1093/cid/civ951>
37. Grant PR, G.J., et al., Detection of SARS coronavirus in plasma by real-time RT-PCR. *N Engl J Med*, 2003; 349:2468-9 <https://doi.org/10.1056/NEJM200312183492522>
38. WHO, WHO recommendations on SARS and blood safety. 2003. <https://www.who.int/csr/sars/guidelines/bloodsafety/en/>.
39. Zhiliang Hu, C.S., Chuanjun Xu, et al., Clinical Characteristics of 24 Asymptomatic Infections with COVID-19 Screened among Close Contacts in Nanjing, China. *medRxiv*, 2020. doi: <https://doi.org/10.1101/2020.02.20.20025619>
40. Bai, Y.Y., Lingsheng & Wei, Tao & Tian,et al., Presumed Asymptomatic Carrier Transmission of COVID-19. *JAMA*, 2020. <https://doi.org/10.1001/jama.2020.2565>
41. Ebrahim SH, Memish ZA. COVID-19: preparing for superspreader potential among Umrah pilgrims to Saudi Arabia. *Lancet*. 2020; 395(10227):e48. [https://doi.org/10.1016/S0140-6736\(20\)30466-9](https://doi.org/10.1016/S0140-6736(20)30466-9)
42. Toru Hifumi, A.Y., Manabu Ato, Clinical Serum Therapy: Benefits, Cautions, and Potential Applications. *Keio J Med*, 2017; 66 (4):57-64. <https://doi.org/10.2302/kjm.2016-0017-IR>
43. Patricia Ribeiro de Carvalho,aPierre Sirois,b and Patricia Dias Fernandes,* The role of kallikrein-kinin and renin-angiotensin systems in COVID-19 infection. *Peptides*. 2021 Jan; 135: 170428. <https://doi.org/10.1016/j.peptides.2020.170428>
44. Floyd RA, Scheider JE, Dittmer DP, methylene blue photoinactivation of RNA viruses. *Antivir Res* 2004; 61:141-151 <https://doi.org/10.1016/j.antiviral.2003.11.004>
45. Bojadzic D, Alcazar O, Buchwald P. Methylene blue inhibits in vitro the SARS-CoV-2 spike - ACE2 protein-protein interaction - A mechanism that can contribute to its antiviral activity against COVID-19 [Internet]. *bioRxiv*. 2020. <https://doi.org/10.1101/2020.08.29.273441>
46. Cagno V, Medaglia C, Cerny A, Cerny T, Tapparel C, Cerny E. Methylene Blue has a potent antiviral activity against SARS-CoV-2 in the absence of UV-activation in vitro [Internet]. 2020. <https://doi.org/10.1101/2020.08.14.251090>
47. Valeria Cagno et al. Methylene Blue has a potent antiviral activity against SARS-CoV2 in the absence of UV-activation in vitro 2020, Aug 14. <https://doi.org/10.1101/2020.08.14.251090>
48. Svyatchenko VA, Nikonov SD, Mayorov AP, Gelfond ML, Loktev VB. Antiviral photodynamic therapy: Inactivation and inhibition of SARS-CoV-2 in vitro using methylene blue and Radachlorin. *Photodiagnosis Photodyn Ther*. 2021; 33(102112):102112. <https://doi.org/10.1016/j.pdpdt.2020.102112>
49. Changzhong Jin et al, Methylene blue photochemical treatment as a reliable SARS-CoV-2 plasma virus inactivation method for blood safety and convalescent plasma therapy for COVID-19. *BMC Infectious Diseases*. 2021; 16:21 <https://doi.org/10.1186/s12879-021-06344-9> Available: <https://bmcinfectdis.biomedcentral.com/articles/10.1186/s12879-021-05993-0>
50. Gliberto L., Pardo A. The rationale for methylene blue utility against SARS-CoV-2 infection Complications.j ppres.2021; 9(3):379-396.
51. Domingo P, Mur I, Pomar V, Corominas H, Casademont J, deBeenlto N, The four horsemen of a viral Apocalypse: The pathogenesis of SARS-CoV-2 infection(covid-19). *EBioMedicine* 2020; 58:102887. <https://doi.org/10.1016/j.ebiom.2020.102887>
52. Ahn H, et al, Methylene blue inhibits NLRP3, NLRC4, AIM2, and non-cononical inflammasome activation. *Sci Rep* 2017; 7(11):2409 <https://doi.org/10.1038/s41598-017-12635-6>
53. Lum H, Roebuck KA. Oxidant stress and endothelial cell dysfunction. *Am J Physiol Cell Physiol* 2001; 280:C719-41 <https://doi.org/10.1152/ajpcell.2001.280.4.C719>
54. Sciglino G, Sciglino GA. Methylene blue in covid 19. *jmedhy*. 2020 Dec 10 ;146 <https://doi.org/10.1016/j.mehy.2020.110455>
55. Denny JT, Burr AT, Balzer F, Tse JT, Denny JE, Chyu D. Methylene blue treatment for cytokine release syndrome-associated vasoplegia following a renal transplant with rATG infusion: A Case report and literature review. *Exp Ther Med* 2015; 9(5):1915-2 <https://doi.org/10.3892/etm.2015.2349>
56. Zhao RZ et al., Mitochondrial electron transport chain, ROS generation and uncoupling (Review). *Int J Mol Med*. 2019 Jul; 44(1):3-15. <https://doi.org/10.3892/ijmm.2019.4188>
57. Salaris SC, Babbs CF, Voorhees III WD, methylene blue as an inhibitor of superoxide generation by xanthine oxidase, *Biochem pharmacol* 1991; 42(3): 499-506. [https://doi.org/10.1016/0006-2952\(91\)90311-R](https://doi.org/10.1016/0006-2952(91)90311-R)
58. Salaris SC, Babbs CF, Voorhees WD, 3Rd; methylene blue as an inhibitor of superoxide generation by xanthine oxidase. A potential new drug for the attenuation of ischemia/reperfusion injury; *Biochem pharmacol*. 1991 Jul 15; 42(3):499-506. [https://doi.org/10.1016/0006-2952\(91\)90311-R](https://doi.org/10.1016/0006-2952(91)90311-R)
59. Moncada S, Palmer RMJ and Higgs EA, Nitric oxide-physiology, pathophysiology, and pharmacology. *pharmacol Rev* 1991; 43:19-142,
60. Mayer B, et al., Biosynthesis of endothelium-derived relaxing factor: a cytosolic enzyme in porcine aortic endothelial cells Ca²⁺-dependently converts L-arginine into an activator of soluble

- guanylyl cyclase. *BiochemBiophys Res Commun* 1989; 164:678-685.
61. Miki N, Kawabe Y and kuriyama K, Activation of cerebral guanylate cyclase by Nitric oxide. *Biochem BIOPHYS Res Commun* 1977; 75:851-856. [https://doi.org/10.1016/0006-291X\(77\)91460-7](https://doi.org/10.1016/0006-291X(77)91460-7)
 62. Willis, R.A., et al., Induction of nitric oxide synthase in subsets of murine pulmonary fibroblasts: effect on fibroblast interleukin-6 production. *ClinImmunol Immunopathol*, 1994; 71(2):231-9. <https://doi.org/10.1006/clin.1994.1077>
 63. Bernd Mayer, Friedrich Brunner and Kurt Schmidt: Inhibition of nitric oxide synthesis by methylene blue, *Biochemical Pharmacology* 1993; 45(2):367-374. [https://doi.org/10.1016/0006-2952\(93\)90072-5](https://doi.org/10.1016/0006-2952(93)90072-5)
 64. Leonard Naymagon , et al, Troy :the emergence of methemoglobinemia amidst the covid-19 pandemic, *American journal hematology*: published on 15 may 2020; <https://doi.org/10.1002/ajh.25868>
 65. Murad F, et al., Guanylate cyclase: activation by azide , Nitro compounds , and hydroxyl radical and inhibition by hemoglobin. *Adv Cyclic Nucleotide Res* 1978; 9:145-158,
 66. hinaFaisal, MD, MRCS, Alexi Bloom, MD, + and A. Osama Gaber, MD, FACS : unexplained Methemoglobinemia in coronavirus disease 2019: A Case Report ; *international anesthesia research society*; july 2020; 14(9). <https://doi.org/10.1213/XAA.0000000000001287>
 67. Drugs.com; Methylene blue[internet],USA[updated.February.18.2019;cited February.8.2021]; Available from: <https://www.drugs.com/monograph/methylene-blue.html#pharmacokinetics>
 68. Choo S.Y, Rapidly rising methemoglobinemia in a patients with severe covid-19 treated successfully with red cell exchange transfusion; *TherApher Dial*. 2020 oct 11; <https://doi.org/10.1111/1744-9987.13598>
 69. Dewachter P, Claudiemoutanfaivre, Philippe Trechot, Jean-Claude Lieu, Paul Michel Mertes. Severe anaphylactic shock with methylene blue instillation. *AnesthAnalg*. 2005; 101:149-50. <https://doi.org/10.1213/01.ANE.0000153497.60047.80>
 70. Clifton, Jack II, Leikin, Jerrold Methylene blue. *America Journal of Therapeutics*. 2003; 10:289-91. <https://doi.org/10.1097/00045391-200307000-00009>
 71. Dalvi P,et al., Intravenous methylene blue as a rescue therapy in the management of refractory hypoxia in COVID-19 ARDS patients: A case series. *Indian J Crit Care Med*. 2021; 25(8):934-8. <https://doi.org/10.5005/jp-journals-10071-23905>
 72. Kidd SA, et al, was DM, Fetal death after exposure to methylene blue dye during mid-trimester amniocentesis in twin pregnancy. *Prenatal diagnosis*.1996 Jan; [https://doi.org/10.1002/\(SICI\)1097-0223\(199601\)16:1<39::AID-PD789>3.0.CO;2-P](https://doi.org/10.1002/(SICI)1097-0223(199601)16:1<39::AID-PD789>3.0.CO;2-P)
 73. Martindale SJ Stedeford JC, Neurological sequelae following methylene blue injection for parathyroidectomy. *Anaesthesia* 2003 OCT; https://doi.org/10.1046/j.1365-2044.2003.03415_23.x
 74. Gillman PK, CNS toxicity involving methylene blue; the exemplar for understanding and predicting drug interactions that precipitate serotonin toxicity. *Journal of psychopharmacology* 2011; march; <https://doi.org/10.1177/0269881109359098>