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Full length article

Application of methylene blue -vitamin C –N-acetyl cysteine for treatment of critically ill COVID-19 patients, report of a phase-I clinical trial☆

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ABSTRACT

COVID-19 is a global catastrophic event that causes severe acute respiratory syndrome. The mechanism of the disease remains unclear, and hypoxia is one of the main complications. There is no currently approved protocol for treatment. The microbial threat as induced by COVID-19 causes the activation of macrophages to produce a huge amount of inflammatory molecules and nitric oxide (NO). Activation of macrophages population into a pro-inflammatory phenotype induces a self-reinforcing cycle. Oxidative stress and NO contribute to this cycle, establishing a cascade inflammatory state that can kill the patient. Interrupting this vicious cycle by a simple remedy may save critical patients' lives. Nitrite, nitrate (the metabolites of NO), methemoglobin, and prooxidant-antioxidant-balance levels were measured in 25 ICU COVID-19 patients and 25 healthy individuals. As the last therapeutic option, five patients were administered methylene blue-vitamin C–N-acetyl Cysteine (MCN). Nitrite, nitrate, methemoglobin, and oxidative stress were significantly increased in patients in comparison to healthy individuals. Four of the five patients responded well to treatment. In conclusion, NO, methemoglobin and oxidative stress may play a central role in the pathogenesis of critical COVID-19 disease. MCN treatment seems to increase the survival rate of these patients. Considering the vicious cycle of macrophage activation leading to deadly NO, oxidative stress, and cytokine cascade syndrome; the therapeutic effect of MCN seems to be reasonable. Accordingly, a wider clinical trial has been designed. It should be noted that the protocol is using the low-cost drugs which the FDA approved for other diseases.
Trial registration number: NCT04370288.

1. Introduction

The rapid spread of the deadly COVID-19 caused by SARS-CoV-2 is currently a nightmare for all the world. COVID-19 is responsible for this catastrophic pandemic disease. The WHO reported that 14% of infected patients have severe disease and require hospitalization, 5% of infected patients have very severe conditions and require intensive care admission (mostly for ventilation) and 4% of infected patients die (Grech, 2020).

The huge efforts have been done for finding the pathophysiology and treatment of critical COVID-19 disease. Many therapeutic procedures have been suggested, with little success. There is no global approved protocol for the treatment of critically ill patients that leads to a very low survival rate and prolonged treatment in ICU. This often no success long ICU care, causes a high burden to health systems even in the most developed and rich countries. The exact mechanism of tissue injury

☆ Novel Treatment COVID-19 Methylene Blue

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remains unclear and the management of patients mainly emphasizes the provision of supportive care, e.g., oxygenation, ventilation, and fluid therapy (Galluccio et al., 2020). A better understanding of the pathogenesis of the critical disease state is crucial to develop rationale-based clinical therapeutic strategies and to determine which subsets of patients are at high risk of the severity of the disease. It is critical to start measuring factors involved in the pathways of disease which can be modulated by the different therapies currently used as a standard of care or in clinical trials.

The SARS-CoV-2 causes the activation of macrophages to produce a huge amount of inflammatory molecules, which cytokine storm syndrome is the main cause of the death in COVID-19 patients (Alunno et al., 2020). Also, it is known in other diseases that overproduction of inducible nitric oxide (iNO) (Miclescu and Wiklund, 2010) and reactive oxygen species (ROS) (Shehat and Tigno-Aranjuez, 2019) happen upon macrophage activation which leads to a vicious cycle of macrophage activation for overproduction of cytokines (Wang and Ma, 2008).

Could NO and oxidative stress play a central role in increasing hypoxia which is one of the main complications in COVID-19 patients? Could this vicious cycle of NO and ROS be interrupted using an effective and intense anti-NO and anti-oxidant therapy?

In medicine, methylene blue (MB, the oxidized form, blue color), but not the reduced form (LMB: Leucomethylene blue, colorless), has been used in different diseases such as malaria, surgery, orthopedics, bacterial, viral infections and et cetera (Hamidi Alamdari et al., 2020).

A probable reason for hypoxia in COVID-19 patients is methemoglobinemia which results from oxidation of the iron contained in hemoglobin from the ferrous to the ferric form. The oxidation is associated with a decrement in the capacity of hemoglobin to carry oxygen (Hamidi Alamdari et al., 2020).

In this study, nitrite, nitrate (the metabolites of NO), methemoglobin (met-Hb) and prooxidant-antioxidant balance (PAB) were estimated as involving factors to intensify hypoxia in ICU patients. Five critically ill COVID 19 patients, after standard cares, characterized to be in the final stage by their physicians, have been administered with MB, vitamin C, and N-acetyl Cysteine as a compassionate therapy, and included to a larger clinical trial that is already running.

2. Material and methods

This study was performed at Mashhad University of Medical Sciences, Mashhad, Iran in 2020, after ethics committee approval (ClinicalTrials.gov Identifier: NCT04370288; April 19, 2020) and taking written informed consent. Also, the clinical trial was applied for registration in IRCT (Trial Id 49,767).

This study performed on 25 healthy individuals and 25 patients with COVID-19 pneumonia which were admitted to ICU with Pa02/Fi02 < 200. For all patients' venues blood gas, CBC, LDH, CRP were measured, and high-resolution computed tomography (HRCT) was done.

2.1. Measurement of nitrite, nitrate in plasma samples

NO, due to its short lifetime (few milliseconds), was determined based on the amount of its oxidation products: nitrite and nitrate. Nitrite and nitrate were measured according to the methods described by Yegın which used Griess reaction assay for plasma samples (Yegın et al., 2015).

2.2. Met-Hb

Fresh blood was collected using EDTA-K2 as an anticoagulant and transferred to the laboratory on ice. Met-Hb was measured according to the method described by Sato (Sato et al., 1981).

2.3. PAB

PAB was measured according to the method described by Alamdari DH (Alamdari et al., 2007).

2.4. Clinical trial patients

As the last therapeutic option, five out of 25 ICU COVID-19 patients were recruited in clinical trial and treatment with MCN. The patients were administered MB (1 mg/kg) along with vitamin C (1500 mg/kg) and N-acetyl Cysteine (1500 mg/kg) orally or intravenously as described for each case.

2.5. Clinical trial criteria

Inclusion criteria were: The confirmed case of Covid-19 (by RT-PCR on the nasopharyngeal swab collected or clinical and HR-CT features) and age above 18 years old. Exclusion criteria were: the history of G6PDH deficiency, severe renal failure, cirrhosis, active chronic hepatitis, patients with a history of an allergic reaction to MB, treatment with immunosuppressive agents, and pregnant or breastfeeding female.

3. Results

Demographic characterizations of patients and healthy individuals as well as laboratory results are presented in Table 1. The data of patients before and after treatment by methylene blue are presented in Table 2.

3.1. Case 1

1. On April 13, 2020, a 49-year-old male was admitted to our ICU because of fever, low level of consciousness, decreased SPO2, and highly purulent tracheal secretion. At admission, the patient had RASS -4 (Richmond Agitation Sedation Score), and under mechanical ventilation with fever, tachycardia, SPO2 86–88%. His blood pressure was in the normal range.
2. He had no co-morbidity in his past medical history
3. The patient was admitted previously on February 15, 2020, to another ICU due to headache, cough, myalgia, fever, and dyspnea which are starting 14 days before admission.
4. Lung HRCT revealed diffuse bilateral ground-glass opacities (GGOs) and consolidation in the peripheral lung regions. Both upper and lower lobes were involved.
5. WBC: 16 × 103/μl with 88% neutrophils and 3.6% lymphocytes, platelets counts: 221 × 103/μl, LDH: 906 IU/l, CRP: 82 mg/dl, D-Dimer: 2078 ng/ml, total bilirubin: 2 mg/dl, AST: 134 IU/l and ALT: 89 IU/l. RT- PCR was positive for SARS-CoV-2.
6. He was treated with azithromycin (500 mg/day), hydroxychloroquine (400 mg stat and 200 mg BD), and meropenem 1 gr TDS.
7. Tracheostomy was done because of the long period of intubation.
8. The culture of tracheal secretion revealed multi-resistance microorganisms such as Acinetobacter and Pseudomonas.

Table 1
Demographic characterizations of patients, healthy individuals (HI), and laboratory results.

	HI (n = 25)	Patients group (n = 25)	*P* value
Age (years)	56.6 ± 11.4	59.9 ± 13.6	0.22
Male/Female	12/13	11/14	0.74
$NO2^-$ (μmol/l)	7.6 ± 3.9	10.7 ± 7.9	0.01 [a]
$NO3^-$ (μmol/l)	22.4 ± 15.3	44.7 ± 30.1	0.002 [a]
Met-Hb (%)	2.5 ± 0.9	16.4* ± 9.1	0.0001 [a]
PAB (HK)	35.8 ± 15.3	88.4* ± 28.4	0.0001 [a]
CRP (mg/dl)	8.7 ± 4.5	94.3* ± 49.5	0.0001 [a]
LDH (U/l)	251.6 ± 139.9	1036.6* ± 348.8	0.0001 [a]

The data are presented as mean ± S.D.
[a] There was a significant difference between patients and HI ($p < 0.05$).

Table 2
The data of 4 patients before and after treatment.

	Before Treatment (n = 4)	After Treatment (n = 4)	*P* value
$NO2^-$ (μmol/l)	2.8 ± 13.1	7.0 ± 1.4	0.009 [a]
$NO3^-$ (μmol/l)	68.2 ± 44.7	40.7 ± 25.2	0.05 [a]
Met-Hb (%)	14.7 ± 2.2	4.5 ± 0.5	0.001 [a]
PAB (HK)	90.5 ± 6.4	51.7 ± 21.7	0.001 [a]
CRP (mg/dl)	99.0 ± 31.0	17.7 ± 2.9	0.005 [a]
LDH (U/l)	859.75 ± 219.6	245.0 ± 100.7	0.002 [a]

The data are presented as mean ± S.D.
[a] There was a significant difference between patients and HI ($p < 0.05$).

9. After three days, because of progressive respiratory distress, Kaletra (lopinavir/ritonavir, 200/50 mg) and hydrocortisone were added to the treatment protocol.
10. Nitrite and nitrate, met-Hb, and PAB were 10.2 μmol/l, 35.1 μmol/l, 14%, and 95 HK, respectively.
11. On May 24, 2020, because of a weak response to antibiotics after 45 days and weaning failure, we started to administer oral MB (1mg/kg) vitamin C (1500 mg), N-acetyl Cysteine (2gr) in 100 ml dextrose for twice a day.
12. There was no side effect and an allergic reaction. After 8–12 h the color of urine became blue or green.
13. On the same day of MB administration, the patient was decannulated and tracheostomy was removed and oxygen therapy was started by a reserve bag mask.
14. Antibiotic therapy was continued and dexamethasone (8 mg QID) was started.
15. The day after starting MB therapy, the patient had a large volume of tracheal secretion from the tracheostomy site, but SPO2 was 90–92% on high flow oxygen.
16. On the second and third days after starting MB therapy, the patient improved significantly and SPO2 to 96 by a simple face mask.
17. From the fourth day after MB therapy, dexamethasone was tapered and patient consciousness was improved, the tracheal secretion was decreased but the patient had a low degree of fever.
18. On the sixth day after starting methylene blue, oxygen therapy was discontinued.
19. On the twenty-third day, he was discharged from ICU.
20. After treatment with MCN, nitrite and nitrate, met-Hb, and PAB were 6.6 μmol/l, 24.30 μmol/l, 4%, and 62 HK, respectively.

3.2. Case 2

1. On May 7, 2020, a 64-year-old female was admitted to ICU due to a decreased level of consciousness and respiratory distress. She was immediately intubated.
2. She had a past medical history of depression
3. Based on the criteria of Yang et al. (2020), the patient had a moderate form of the disease due to the presence of fever, respiratory symptoms, and radiological signs of pneumonia.
4. Lung HRCT revealed ground-glass opacities (GGOs) in her left lung.
5. WBC: 12 × 103/μl with 78% neutrophils and 12.5% lymphocytes, platelets counts: 196 × 103/μl, LDH: 670 IU/l, CRP: 79 mg/dl, D-Dimer: >5000 ng/ml, total bilirubin: 1 mg/dl, AST: 116 IU/l and ALT: 76 IU/l and SPO2 96%. RT- PCR was positive for SARS-CoV-2.
6. Treatment was initiated with azithromycin (500 mg/day), hydroxychloroquine (400 mg stat and 200 mg BD), and ceftriaxone 1 gr BD.
7. Nitrite and nitrate, met-Hb, and PAB were 17 μmol/l, 24.3 μmol/l, 16%, and 84 HK, respectively.
8. On May 11, 2020, MB (1mg/kg/) vitamin C (1500 mg), N-acetyl Cysteine (2gr) were added in 100 ml dextrose and administered via nasogastric tube q12 h for 7 days.
9. There were no side effects and an allergic reaction. After 8–12 h the color of urine became blue or green.
10. On the second day, after MB therapy, the patient was afebrile.
11. In the fifth day of MB therapy, the patient was discontinued from mechanical ventilation and extubated.
12. In the ninth day, she was discharged from ICU.
13. After treatment with MCN, nitrite and nitrate, met-Hb, and PAB were 8.5 μmol/l, 18.9 μmol/l, 5%, and 53 HK, respectively.

3.3. Case 3

1. On May 1, 2020, a 60-year-old female was admitted to ICU due to fever and respiratory distress.
2. Based on the criteria of Yang et al. (2020), the patient had a moderate form of the disease due to the presence of fever, respiratory symptoms, and radiological signs of pneumonia.
3. She had a past medical history of diabetes mellitus.
4. Lung HRCT revealed bilateral ground-glass opacities (GGOs).
5. WBC: 8.9 × 10^3/μl with 79% neutrophils and 13% lymphocytes, platelets counts: 275 × 10^3/μl, LDH: 712 IU/l, CRP: 90 mg/dl, D-Dimer: 1508 ng/ml, total bilirubin: 1.8 mg/dl, AST: 125 IU/l and ALT: 95 IU/l. RT- PCR was negative for SARS-CoV-2.
6. Treatment was initiated with azithromycin (500 mg/day), hydroxychloroquine (400 mg stat and 200 mg BD), and ceftriaxone 1 gr BD.
7. Nitrite and nitrate, met-Hb, and PAB were 12 μmol/l, 109.2 μmol/l, 12%, and 86 HK, respectively.
8. On May 4, 2020, MB (1 mg/kg) vitamin C (1500 mg), N-acetyl Cysteine (2gr) in 100 ml dextrose were injected intravenously over 30 min and continued every 12 h for 2 days.
9. There was no side effect and an allergic reaction. After 8–12 h the color of urine became blue or green.
10. On the second day, after MB therapy, SPO2 raised from 84% to 93% after that patient was under oxygen therapy by a simple face mask.
11. On the fourth day of MB therapy, the patient was discharged from ICU.
12. After treatment with MCN, nitrite and nitrate, met-Hb, and PAB were 7.8 μmol/l, 74.5 μmol/l, 5%, and 71 HK, respectively.

3.4. Case 4

1. On April 22, 2020, a 66-year-old male was admitted to the internal ward of the hospital due to fever and respiratory distress.
2. He had no past medical history of the disease.
3. Lung HRCT revealed diffuse bilateral ground-glass opacities (GGOs) and consolidation.
4. WBC: 5.4 × 10^3/μl with 59% neutrophils and 34% lymphocytes, platelets counts: 154 × 10^3/μl, LDH: 906 IU/l, CRP: 124 mg/dl, D-Dimer: 1405 ng/ml, total bilirubin: 0.6 mg/dl, AST: 37 IU/l and ALT: 92 IU/l, SPO2 88%. RT- PCR was positive for SARS-CoV-2.
5. Treatment was initiated with azithromycin (500 mg/day), hydroxychloroquine (400 mg stat and 200 mg BD), and ceftriaxone 1 gr BD.
6. After three days, because of progressive respiratory distress, Ribavirin, Kaletra, and dexamethasone were added to the treatment protocol.
7. After four days of admission, the patient was transferred to ICU and received one-time plasma therapy.
8. One week after plasma therapy, patient oxygenation status was worsening (SPO2 59% with non-invasive ventilation and 100% FIO2)

9. Nitrite and nitrate, met-Hb, and PAB were 45.1 μmol/l, 89.2 μmol/l, 22%, and 100 HK respectively.
10. MB (1 mg/kg) vitamin C (1500 mg), N-acetyl Cysteine (2gr) were added in 100 ml dextrose and prescribed intravenously. Before injection SPO2 was 54%, and after 30 min injection, SPO2 improve to 74% and last for 12 h. Because of unexpected limitations for the preparation of methylene blue, the drug was not continued.
11. There was no side effect and an allergic reaction.
12. On the second day, this patient encountered severe septic shock, multi-organ failure, and was expired. This scenario may be due to a late and incomplete dose of methylene blue.

3.5. Case 5

1. On May 13, 2020, a 75-year-old female had an emergency operation for coronary artery bypass grafting and two days later she showed a decrease in SPO2 and respiratory distress.
2. Based on the criteria of Yang et al. (2020), the patient had a severe form of the disease due to the presence of fever, respiratory symptoms, and radiological signs of pneumonia.
3. Lung HRCT revealed ground-glass opacities (GGOs). She had a combination of consolidation and GGOs. The distribution of abnormalities was bilateral in the subpleural lung regions. Both upper and lower lobes were involved.
4. She had past medical history of diabetes and hypertension.
5. WBC: 8.9 × 10^3/μl with 80% neutrophils and 12.6% lymphocytes, platelets counts: 276 × 10^3/μl, LDH: 1151 IU/l, CRP: 145 mg/dl, D-Dimer: 1374 ng/ml, total bilirubin: 1.5 mg/dl, AST: 115 IU/l and ALT: 90 IU/l. RT- PCR was negative for SARS-CoV-2.
6. On May 15, 2020, she transferred to ICU, she had a stable hemodynamic. SPO2 was 65–68% without oxygen therapy and 78–80% with oxygen therapy via a reserve bag.
7. After stating non-invasive ventilation (NIV) SPO2 reached 87–88%.
8. Treatment was initiated with azithromycin (500 mg/day), lopinavir/ritonavir (200/50 mg, 2 tablets × b.i.d), hydroxychloroquine (400 mg stat), heparin 5000 IU (q.i.dintravenously)., hydrocortisone (starting dose 100 mg TDS, lately tapered) and continued for five days.
9. Nitrite and nitrate, met-Hb, and PAB were 13.5 μmol/l, 104.3 μmol/l, 17%, and 97, respectively.
10. On May 16, 2020, MB (1mg/kg) vitamin C (1500 mg), N-acetyl Cysteine (2gr) were added in 100 ml dextrose and prescribed orally.
11. There was no side effect and an allergic reaction. After 8–12 h the color of urine became blue or green.
12. Due to heart operation and leukocytosis, the prophylactic antibiotic was administrated.
13. At the first day, after MB therapy, there was no significant change in SPO2
14. On the second day of MB therapy SPO2 increased and the duration of noninvasive ventilation (NIV) was decreased.
15. On the fourth day of MB therapy, the patient did not need NIV, and SPO2 was 80–82% without oxygen therapy and reached 97–99% with oxygen therapy.
16. On the fifth day of MB therapy, the patient did not need oxygen therapy and SPO2 was 90–92% and she was completely awake and oral feeding was started.
17. On the seventh day, she was discharged from ICU.
18. After treatment with MCN, nitrite and nitrate, met-Hb, and PAB were 5.1 μmol/l, 45.2 μmol/l, 4%, and 21, respectively.

4. Discussion

This study showed that nitrite, nitrate, met-Hb, and oxidative stress are significantly increased in patients in comparison to healthy individuals. This is compatible with the inflammatory process and macrophage activation that has been observed in COVID 19 disease (Alunno et al., 2020; Wang and Ma, 2008).

The rationale for considering these factors involving in the pathophysiology of inducing hypoxia are as following:

1 The microbial threat is induced by coronavirus causes the activation of macrophages to produce a huge amount of inflammatory mediators and other molecules such as a nitric oxide (NO). NO acts as a pro-inflammatory and an anti-inflammatory agent, depending on the amount of NO generation and its source (Kobayashi and Murata, 2020). It is documented that macrophage inducible nitric oxide synthase (iNOS) circulates inflammation (Wang et al., 2018). It is reported that following cytokine administration, increased levels of nitrite has been detected in many diseases such as sepsis, ulcerative colitis, arthritis, multiple sclerosis, and type I diabetes (Hibbs et al., 1992). Also, excessive NO is produced during the course of a variety of inflammatory diseases (Clancy and Abramson, 1995). It is reported that there is a key role for monocytes and macrophages for pathological inflammation; and there is an ongoing discussion about the prospective therapeutic strategies to modulate macrophage activation in patients with COVID-19 (Merad and Martin, 2020).

2 Our results showed increase levels of nitrite and nitrate of blood in COVID-19 patients. Macrophage inducible nitric oxide synthase (iNOS) can be induced 2 to 3 orders of magnitude following inflammation which releases large amounts of NO leading to local and systemic increases of nitrite. (Kleinbongard et al., 2003).

3 Our results also showed increased levels of oxidative stress in COVID-19 patients. NO is one of the major resources of oxidative/nitrosative stress. NO can react with reactive oxygen species such as superoxide ($O2^{\bullet -}$), to form peroxynitrite ($ONOO^-$), a highly reactive species which damages cells. On the other hand, COVID-19-associated coagulopathy is reported as the occurrence of venous and arterial thrombotic events including deep vein thrombosis (DVT), pulmonary embolism (PE), ischemic stroke, myocardial infarction (as it probably observed in our case of No 5), and systemic arterial events (Becker, 2020). Nitric oxide (NO), which is generated by iNOS, is regarded as a critical mediator of coagulation abnormalities and organ dysfunction. . It is proved that selective iNOS inhibition is associated with attenuation of sepsis-induced coagulation and endothelial dysfunction. The reduction of oxidative/nitrosative stress probably contributes to the beneficial effects afforded by iNOS blockade (Matejovic et al., 2007). Numerous studies have revealed that both thrombus formation and its clot lysis regulated by oxidative stress. After resolution of deep vein thrombosis, D-Dimer will be increased and oxidative stress could promote the resolution (Gutmann et al., 2020). We saw increased oxidative stress and D-Dimer in our cases.

4 NO is rapidly oxidized to nitrite in blood by ceruloplasmin. Nitrite and nitrate are considered as the marker of NO generation and a relatively inert end product of NO metabolism (Shiva et al., 2006). There is a significant correlation between CRP and nitrite in inflammatory diseases. It is suggested that the measurement of nitrite could be a diagnostic, as well as prognostic tool, during the treatment of this disease (Ersoy et al., 2002). We also observed an increase in nitrite and CRP levels in the patients probably secondary to increase oxidative stress.

5 Nitrite passes through the RBC membrane. The nitrite anion is known to oxidize Hb to Met-Hb and also degrade Hb which resulted in increasing hypoxia, bilirubin, and iron. Met-Hb plays a central role in inducing more hypoxia and correction of Met-Hb may be a critical point for treatment (Vitturi et al., 2009). In this study, met-Hb was significantly increased in patients in comparison to healthy individuals, which causes hypoxemia. Treatment of the patients with

MB probably decreased the met-Hb and subsequently hypoxemia levels, as we observed in our case reports.

6 In addition to NO, oxidative stress also leads to oxidation of hemoglobin (Fe^{2+}) to met-Hb (Fe^{3+}) (Gutmann et al., 2020). Our results showed the increase of oxidative stress and met-Hb in patients.

7 Oxidative stress and inflammation simultaneously interact with each other and exacerbate their effects by creating a vicious cycle to worsen the diseases by reactive oxygen and nitrogen species (ROS and RNS) over-production which contribute to organ damage by oxidation and nitrosation of various biological targets and components of the cell including lipids, thiols, amino acid residues, DNA bases, and low-molecular-weight antioxidants (Yegın et al., 2015). As we mentioned, MB reduction turns the blue color form to colorless one. After 8–12 h of consumption, we observed the color of urine turned to blue or green. This shows probably that the oxidant agents in blood oxidase the reduced form of MB (colorless) to the oxidized form (blue color). This chemical reaction probably decreases the oxidative stress and subsequently inflammatory mediators.

8 Evidence has shown that elderly people and those with pre-existing multi-morbid conditions may be at higher risk of developing severe health consequences from COVID-19. “Oxi-inflamm-aging” refers to the phenomenon of chronic low-grade systemic inflammation that accompanies aging. Aging cells have a decreased ability to proliferate which this senescence cell stimulates the secretion of pro-inflammatory cytokines that cause chronic inflammation independent from the activation of immune cells. This inflammation also leads to increased levels of ROS and RNS which could induce oxidative/nitrosative stress. Oxidative/nitrosative stress can also lead to the activation of pro-inflammatory pathways in the body, contributed to the pathogenesis of many age-related diseases. Therefore, age-related Inflammation exacerbates the production of NO (by iNOS), which induces a highly oxidative/nitrosative stress (Matsushita et al., 2020).

In our recent suggested protocol (Hamidi Alamdari et al., 2020), we explained in detail the rationale behind using MB, vitamin C, and N-acetyl Cysteine for the treatment of patients in the clinical trial, and we will explain other reasons in following:

(1) It is documented that MB has direct inhibitory effects on nitric oxide synthases (NOS), both constitutive and inducible, and prevent the accumulation of cyclic guanosine monophosphate (cGMP) by inhibiting the enzyme guanylate cyclase (Miclescu and Wiklund, 2010). In a clinical study, it is documented that NO is a potential mediator of the hemodynamic changes associated with sepsis. The adverse effect of NO on hemodynamic can be partially antagonized by methylene blue, through inhibition of the enzyme guanylate cyclase (Brown et al., 1996).

(2) MB increases the activity of normally slow NADPH–methemoglobin reductase pathway, which decreasing hypoxia through reducing met-Hb. A small amount of met-Hb is always being formed but is reduced within the erythrocyte by these enzymes: (1) NADH cytochrome-b5 reductase, (2) NADPH–methemoglobin reductase. One of FDA treatment for methhemoglobinemia is the application of MB (1–2 mg/kg IV over 5–30 min) and other treatments are ascorbic acid and reduced glutathione (McPherson, 2017).

(3) Increased levels of methemoglobin are secondary to (A) decreased hereditary or acquired NADH cytochrome-b5 reductase activity; in the homozygote NADH-cytochrome-b5 reductase deficiency, met-Hb levels are 10%–50% (cyanotic). Met-Hb concentrations of 10%–25% may give no apparent symptoms; levels of 35%–50% result in mild symptoms, such as exertional dyspnea and headaches; and levels exceeding 70% are probably lethal; (B) increased acquire production of met-Hb can be induced by drugs or chemical agents such as nitrites, nitrates, chlorates, quinones, and aromatic amino and nitro compounds.[26] The mean met-Hb level in our 25 ICU patients was increased (Table 1).

(4) We hypothesized (Wang and Ma, 2008) that reduced form of MB (Leucomethylene: LMB) can also decrease the methemoglobinemia in COVID-19 patients by these mechanisms: (A) Rapid direct effect: reducing the met-Hb (as we saw in case 4); (B) Decreasing oxidative stress: LMB, as a reducing agent, quench ROS, however MB (the oxidized form) induces oxidative stress through absorbing electron (like a free radical) from other molecules ($NADH\text{-}H^+$, $NADPH\text{-}H^+$, GSH), and then decreases met-Hb through enzymatic mechanism (McPherson, 2017). Therefore, we used the reduced form of MB which could not induce oxidative stress. (C) Decreasing the inflammation: This reduces oxidative stress and vice versa. Experimental and clinical studies have also been shown that MB decreases inflammation (Shehat and Tigno-Aranjuez, 2019).

(5) MB could prohibit the cytopathic effect and reduce the propagation of RNA virus (such as poliovirus) through these ways: (1) mechanical effect by the easily penetrating MB which could competitively occupy cellular sites necessary for virus attachment, penetration and/or multiplication; (2) Decreasing oxidative stress by uncoupling oxidation and phosphorylation; (3) virucidal effect of MB, a lipophilic substance, by entering into the virus through lipid membrane and binding to RNA (Kovács, 1960).

(6) Antibacterial properties: MB has formed the basis of antimicrobial chemotherapy-particularly in the area of antimalarials – and the neuroleptic drug families. It is used in an antibacterial foam dressing for the management of chronic wounds with local infection (Woo and Heil, 2017).

(7) MB is a powerful oxygen superoxide scavenger that eliminates rapidly this ion not to damage the tissue. This anion is produced during ischemia-reperfusion in conditions such as acute myocardial infarction and et cetera. (Wülfert et al., 2003). Another antioxidant effect of MB is through blocking the iron-containing enzymes such as xanthine oxidase which prevent to produce ROS (Miclescu and Wiklund, 2010).

(8) MB prevents platelet activation, adhesion, and aggregation through inhibiting the arachidonic acid metabolism in platelets (Miclescu and Wiklund, 2010). This is very important in COVID-19 patients since one of the main complications is thrombotic events (Becker, 2020).

5. Conclusion

Preliminary results of this clinical trial showed the treatment of severe COVID-19 with a mixture of MB, vitamin C, and N-acetyl Cysteine is safe and feasible. The reduced MB has rapid and delayed effects. The rapid effect increases the SPO2% (All patients have been received 100% oxygen) by reducing met-Hb. Delayed effects are through the acceleration of normally slow NADPH–methemoglobin reductase, the improvement of inflammatory markers such as CRP level and LDH, decreasing severity of disease that may be also due to antimicrobial effect. We suggest the optimal time of reduced methylene blue (LMB) administration should be before entering the patient to a very severe stage of the disease and multi-organ involvement and failure. It is the opinion of the authors that the observed results if verified in more patients and a randomized multicenter clinical trial could significantly reduce the mortality of COVID-19 infection and ICU stay average length.

Ethical approval

IR.MUMS.REC.1399.122; Clinical Trials.gov Identifier: NCT04370288; April 19, 2020.

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Research data

Any physician desire to run a randomized clinical trial as a multi-center trial, the authors keen to share their experiences and the last update of their information.

CRediT authorship contribution statement

Daryoush Hamidi Alamdari: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. **Ahmad Bagheri Moghaddam:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Visualization, Writing - original draft, Writing - review & editing. **Shahram Amini:** Conceptualization, Data curation, Investigation, Validation, Visualization, Writing - original draft, Writing - review & editing. **Mohammad Reza Keramati:** Conceptualization, Data curation, Methodology, Writing - original draft, Writing - review & editing. **Azam Moradi Zarmehri:** Conceptualization, Data curation, Methodology, Writing - original draft, Writing - review & editing. **Aida Hamidi Alamdari:** Data curation, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Mohammadamin Damsaz:** Data curation, Methodology, Visualization, Writing - original draft, Writing - review & editing. **Hamed Banpour:** Formal analysis, Software, Visualization, Writing - original draft, Writing - review & editing. **Amir Yarahmadi:** Funding acquisition, Methodology, Validation, Visualization, Writing - original draft, Writing - review & editing. **George Koliakos:** Conceptualization, Data curation, Methodology, Writing - original draft, Writing - review & editing.

Declaration of competing interest

There are no conflicts of interest in all authors.

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