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Review

# Implications of SARS-Cov-2 infection on eNOS and iNOS activity: Consequences for the respiratory and vascular systems

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ABSTRACT

Symptoms of COVID-19 range from asymptomatic/mild symptoms to severe illness and death, consequence of an excessive inflammatory process triggered by SARS-CoV-2 infection. The diffuse inflammation leads to endothelium dysfunction in pulmonary blood vessels, uncoupling eNOS activity, lowering NO production, causing pulmonary physiological alterations and coagulopathy. On the other hand, iNOS activity is increased, which may be advantageous for host defense, once NO plays antiviral effects. However, overproduction of NO may be deleterious, generating a pro-inflammatory effect. In this review, we discussed the role of endogenous NO as a protective or deleterious agent of the respiratory and vascular systems, the most affected in COVID-19 patients, focusing on eNOS and iNOS roles. We also reviewed the currently available NO therapies and pointed out possible alternative treatments targeting NO metabolism, which could help mitigate health crises in the present and future CoV's spillovers.

## 1. Introduction

At the end of 2019, a new coronavirus (CoV), the severe acute respiratory syndrome (SARS)-CoV-2 emerged in Wuhan, China [1]. SARS-CoV-2 is the causative virus of coronavirus disease 2019 (COVID-19), whose symptoms range from asymptomatic to severe respiratory failure, leading patients to hospitalization and even to death [2]. Although seven known CoVs have crossed the species barrier and are endemic in humans, CoVs are broadly distributed in wildlife [3]. The manifold reports detecting numerous CoVs in animals, coupled with the ongoing and pre-existed spillovers of coronavirus in humans, indicate that future zoonotic transmission events may occur [4–7].

In the light of the new CoV outbreak, researchers around the world are making a task force to develop therapeutic strategies, targeting on the virus itself and the disease caused by the SARS-CoV-2 [8]. Although some infected individuals have no symptoms, others report from common cold to severe respiratory failure, such as acute respiratory distress syndrome (ARDS), with multiple organ failure, as a consequence of an excessive inflammatory process triggered by SARS-CoV-2 infection [9]. The diffuse inflammation causes pulmonary physiology damage, and also endothelial disfunction and disturbances in nitric oxide (NO) metabolism, which activate coagulation and thrombin generation leading to thrombosis and pulmonary embolism observed in severe COVID-19 patients [10]. Attempting to find an immediate solution for COVID-19, approved drugs by the Food and Drug Administration (FDA) and similar agencies to treat other diseases are being investigated [11–13]. Nevertheless, there is no effective treatment for COVID-19, the strategies are to try minimizing the chances of hospitalization in the intensive care unit and to avoid invasive procedures like extracorporeal membrane oxygenation (ECMO) to oxygenate the lungs [13].

In this sense, inhaled NO, a non-invasive method, has been investigated to treat COVID-19 and reduce the need for invasive mechanical ventilation. In addition to being a pulmonary vasodilator, NO can act as an anti-inflammatory and antithrombotic agent. NO donors and NO gas also showed antibacterial and antiviral properties in *in vitro* studies and early clinical investigations [14]. However, excessive exposure at high levels of NO can be deleterious to the organism; cells in pro-oxidative state use NO to produce toxic metabolites known as reactive nitrogen oxide species [15], that might aggravate the problems associated with COVID-19 [16]. Here, we reviewed the role of NO in protecting or damaging of the respiratory and vascular systems, the most affected in COVID-19, pointing to possible therapies targeting NO metabolism.

## 2. Metabolism of nitric oxide in the ARDS context

NO is generated along with L-citrulline from L-arginine and oxygen

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($O_2$) in a reaction catalyzed by NO synthase (NOS). Of the three known isoforms of NOS, neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutively expressed NOS (cNOS), while the expression of inducible NOS (iNOS) depends on inflammatory stimuli (reviewed in Ref. [17]). Evidence demonstrates that for NO production, iNOS preferably uses cytosolic L-arginine [18] and cNOS depends on a compartmentalized pool of L-arginine produced from L-citrulline recycling via the citrulline-NO cycle [19]. Therefore, arginosuccinate synthase (ASS), the rate-limiting enzyme in this cycle [20] tightly controls cNOS-produced NO, whereas iNOS produces high levels of NO for hours or days since cytosolic L-arginine concentration is not limited and is above the Km of the NOS [19,21]. However, high levels of NO can be deleterious to the organism; cells in pro-oxidative state use NO to produce reactive species [22]. In this case, the arginase, enzyme that catabolizes L-arginine, is activated to reduce iNOS-produced harmful NO, interfering in beneficial NO production as it also competes with cNOS for this substrate [23]. Low L-arginine availability is associated with ARDS, and may also lead to eNOS uncoupling, which induces further oxidative and cellular damage in the pulmonary epithelium and endothelium [24–26]. For all these reasons, it is of particular interest to ensure the NO production at a safe level, so that the deleterious threshold is not exceeded. In this sense, keeping a pool of L-arginine directed to NO production by the L-citrulline recycling would be a strategy to treat diseases that involve dysregulation of NO metabolism [15].

## 3. The importance of eNOS activity in the pathogenesis of SARS-CoV-2

### 3.1. NO and pulmonary physiology

The most prevalent symptoms of COVID-19 are fever, cough and fatigue, however, about 20% of patients may experience more severe symptoms associated with ARDS [27,28]. ARDS is an acute diffuse inflammatory lung injury, which drives to increased pulmonary vascular permeability. Physiological alterations caused by the infiltration of immune cells lead to greater venous admixture (wasted perfusion), increased physiological dead space and decreased lung compliance [29]. Chest computed tomography (CT) scans from COVID-19 patients show the presence of ground-glass opacity and consolidation since early phase of the disease. Linear opacities, crazy-paving pattern, reverse halo sign and other alterations were observed in patients in the late phase. Also, lung tissue involvement is not homogeneous, which means that patients have different parts of the lungs compromised [30–32]. These findings confirm that the virus causes physiopathological alterations in lung parenchyma and pulmonary vascular structure, which induce ARDS.

ARDS patients are divided into three exclusive categories (mild, moderate, and severe) based on degree of hypoxemia, which is primarily caused by ventilation-perfusion rate (V/Q) mismatch, an outcome of pulmonary vascular changes [29,33]. Dual-energy CT imaging of COVID-19 pneumonia has shown a higher perfusion in areas with opacities and consolidation, lowering the V/Q and causing venous admixture [34]. In healthy individuals, hypoxemia induces vasoconstriction in intrapulmonary arteries to reduce desaturated blood flow through underventilated areas in the lungs, redirecting it to better-oxygenated lung segments [35,36]. Inversely, high blood pressure in these specific ventilated areas of the lungs promotes shear stress, which increases eNOS activity and, hence, NO-induced vasodilatation [37] (Fig. 1A). However, ARDS diffuse inflammatory process triggers a vasodilatation cascade in non-ventilated parts of the lungs (deregulating hypoxic vasoconstrictive mechanisms) and vasoconstriction in ventilated areas. The imbalance between vasoconstricting and vasodilating pathways leads to endothelium dysfunction (Fig. 1B) [34,35,38].

The diffuse inflammation in lung tissue stimulates phenotypic changes in blood vessel endothelial cells, due to injury and increased arginase activity, lowering L-arginine availability, which leads to eNOS uncoupling, increasing RNOS production and contributing to endothelial dysfunction [24,26,39,40]. These abnormalities together suggest an intrapulmonary shunt to areas where gas exchange is compromised, worsening V/Q mismatch, causing hypoxemia, increased vascular pulmonary resistance and pulmonary hypertension [41].

Evidence from post-mortem samples indicates that COVID-19-induced endothelial dysfunction can be caused not only in an indirect manner, by the recruitment of immune cells induced by SARS-CoV-2 infection of susceptible cell types, but also in a direct manner, through endothelial cell infection [42,43]. After binding with angiotensin-converting enzyme 2 (ACE2), the virus enters the cell mainly by

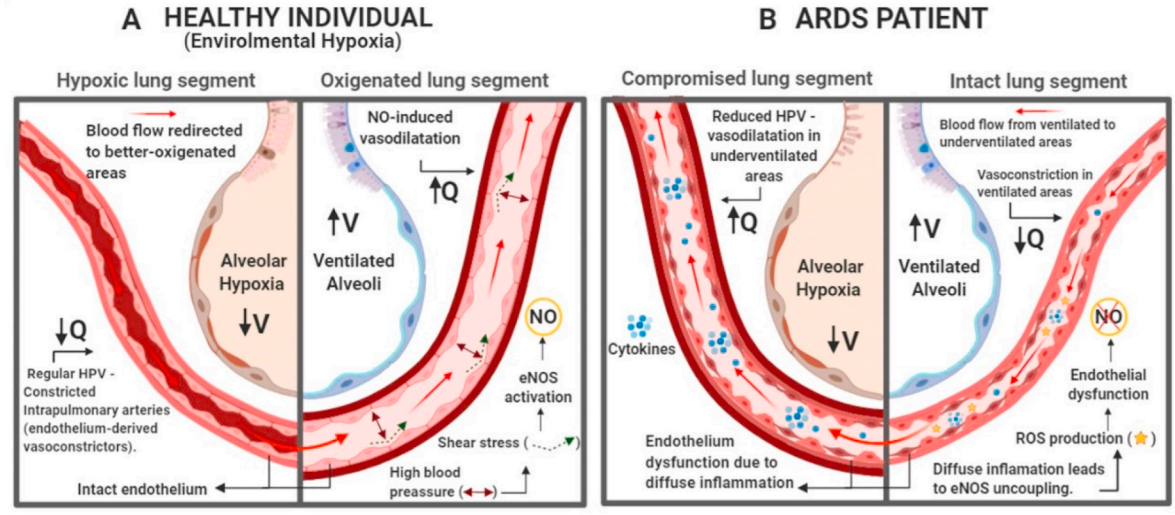


**Fig. 1.** Implications of ARDS in hypoxic pulmonary vasoconstriction (HPV). **(A)** In individuals with intact endothelium, alveolar hypoxia induces vasoconstriction in intrapulmonary arteries, redirecting blood flow to well ventilated areas. The blood pressure in arteries near ventilated alveoli rises, which in turn promotes shear stress, induces eNOS activity and increases the concentration of endothelium-derived vasodilators, like NO, inhibiting HPV and promoting widening of vessel diameter. This regulation causes the blood to flow in direction of well-ventilated areas, improving V/Q [35–37]. **(B)** In ARDS patients, the diffuse inflammation causes endothelial dysfunction in intrapulmonary arteries, causing reduction of HPV. In this situation, the production of endothelium-derived vasoconstrictors (endothelin and thromboxane) is disrupted, causing relaxation of vessel walls in underventilated areas. Once the blood flow is not redirected to well ventilated areas and the activation of eNOS by shear does not occur, inhibiting the production of endothelium-derived vasodilators. These events may cause redirection of blood flow to areas were gas exchange is compromised, worsening V/Q [34,38]. Created with BioRender.com.

endocytosis. ACE2 is internalized and downregulated on endothelial cells, causing renin-angiotensin system (RAS) imbalance. Once ACE2 expression is diminished on endothelial cells, the generation of angiotensin-1-7 (Ang 1-7) is reduced, decreasing the activation of MAS receptor, lowering the release of NO from endothelial cells, causing vasoconstriction, platelet aggregation and disruption of cell-autonomous immunity [12,44,45]. The endothelial barrier importance is such that the elderly and patients with pre-existing risk factors who have compromised endothelium are more susceptible to severe COVID-19 (Table 1).

In an attempt to reverse severe ARDS symptoms and prevent progression in COVID-19 early phase patients, many therapeutic options that modulate respiratory physiology are being proposed, like plateau airway pressure, neuromuscular blockade, extracorporeal membrane oxygenation and inhaled NO [56]. Up to now, inhaled NO is under study in dozens of CoV-related clinical trials [57] These interventional studies not only focus on reversing virus burden and respiratory failure in patients on mechanical ventilation, but also treat and prevent progression in patients with mild and moderate disease and as a preventive option for healthcare providers [57]. Under physiological conditions, blood vessel endothelial cells produce NO, which diffuses into the smooth muscle layer and promotes relaxation and vasodilation through physiological activation of NO-sensitive guanylyl cyclase forming cGMP from GTP. cGMP plays a key role in maintaining the physiological tissue homeostasis, regulating the activity of different targets downstream, such as cGMP-regulated ion channels, cGMP-dependent phosphodiesterases and cGMP-dependent protein kinase (PKG) [58]. After activation by cGMP, PKG phosphorylates myosin light chain phosphatase, which in turn dephosphorylates regulatory light chain (RLC) of myosin, promoting relaxation. cGMP also decreases $Ca^{2+}$ cytosolic concentration, inhibiting the $Ca^{2+}$/calmodulin-dependent protein kinase, myosin light chain kinase, activity, therefore preventing RLC phosphorylation and contributing to smooth muscle relaxation [59]. Given that eNOS is uncoupled in patients with blood vessel endothelial dysfunction, inhaled NO has been used as therapeutic option to replace the endogenous NO activity in patients with several pulmonary complications, including ARDS. As inhaled NO acts selectively, only inducing vasodilatation in lung areas where ventilation is not compromised, it can improve V/Q mismatch [60,61].

Nevertheless, inhaled NO application remains debatable. Inhaled NO benefits on oxygenation is transitory and does not appear to be associated with increased survival. Likewise, most ARDS patients die from multiple organ failure rather than hypoxemia [62]. Moreover, prolonged exposure to inhaled NO can cause sensitization, lowering the oxygenation benefit while exposing these patients to oxidative toxic damage, reducing its benefits [63,64]. Renal function in patients receiving inhaled NO treatment can also be compromised, increasing the need for renal replacement therapy [65]. In order to avoid complications, controlled therapies to regulate the metabolism of NO should be investigated, including an allosteric ASS activator, the step-limiting enzyme of NO-citrulline cycle [15,66].

### 3.2. Coagulation pathway and endothelial dysfunction in COVID-19

Besides the deleterious effects of SARS-CoV-2-induced diffuse inflammation in pulmonary physiology and oxygen saturation, such virus can also induce coagulopathy. Autopsies from COVID-19-positive patients have shown diffuse alveolar damage, widespread lung vascular thrombosis, microvascular thromboembolic, capillary congestion and deep venous thrombosis [67]. Thus, high d-dimer levels in the blood, a coagulopathy marker, are associated with increased mortality in COVID-19 patients. Indeed, pulmonary embolism was the direct cause of death in some patients [42,67,68]. Multi-organ failure, observed in severe COVID-19 cases, has been linked with diffuse intravascular coagulation and large-vessel thrombosis [42,69,70]. Therefore, National Institute of Health treatment guideline for COVID-19 patients recommends anticoagulant therapy as prophylaxis for hospitalised individuals [71].

In the COVID-19, several factors contribute for coagulopathy. The inflammatory response, generated by virus infection, leads to the activation of coagulation cascade, thrombin generation and fibrinolysis shutdown [72–74]. Hypoxemia can also contribute to coagulopathy, increasing blood viscosity and triggering the release of hypoxia-inducible transcription factors, that in turn influence the coagulation and fibrinolysis cascades [75]. Furthermore, endothelial injury and dysfunction caused by pro-inflammatory cytokines and

**Table 1**
Pre-existing alterations in endothelium as risk factors for severe COVID-19.

| Risk factors | Pre-existing conditions | COVID-19 Complications |
|---|---|---|
| Old age [46,47] | Age-related physiological changes:<br>• Age-dependent decreases in NO bioavailability.<br>• Increased oxidative stress.<br>• Increased inflammation.<br>• Reduced shear-stress response<br>• Reduced Tetrahydrobiopterin availability. | • Weak endothelium, more susceptible to SARS-CoV-2 infection.<br>• Exacerbation of endothelial dysfunction and its consequences. |
| Pregnancy [48,49] | Physiological adaptations in pregnancy:<br>• Vascular remodeling.<br>• Overexpression of ACE2.<br>• Increase production of Ang 1–7.<br>• Increased endothelial NO release.<br>• Vasodilatation<br>• Decreased blood pressure | • Greater risk for SARS-CoV-2 infection.<br>• Downregulation of ACE2 - unbalanced vasodilatory responses.<br>• Increased blood pressure.<br>• High risk of preeclampsia. |
| Diabetes [50–53] | • Mitochondrial collapse – ROS production<br>• Reduced eNOS expression.<br>• Impairment of endothelial repair<br>• Reduced Tetrahydrobiopterin availability<br>• Increased arginase activity<br>• Low ACE2 expression | • Exacerbation of endothelial dysfunction and its consequences.<br>• Worsening of dysglycemia<br>• Aggravation of RAS imbalance<br>• Pancreatic b-cells damage |
| Cardiovascular diseases [54,55] | • Endothelial cells in a pro-inflammatory and pro-thrombotic state.<br>• Elevated ROS production.<br>• Reduced NO bioavailability. | • Myocardial injury.<br>• Arrhythmia.<br>• Acute cardiac injury. |

*Abbreviations*: NO, nitric oxide; ACE2, angiotensin-converting enzyme 2; Ang 1–7, angiotensin-1-7; ROS, reactive oxygen species; eNOS, endothelial nitric oxide synthase; RAS, renin-angiotensin system.

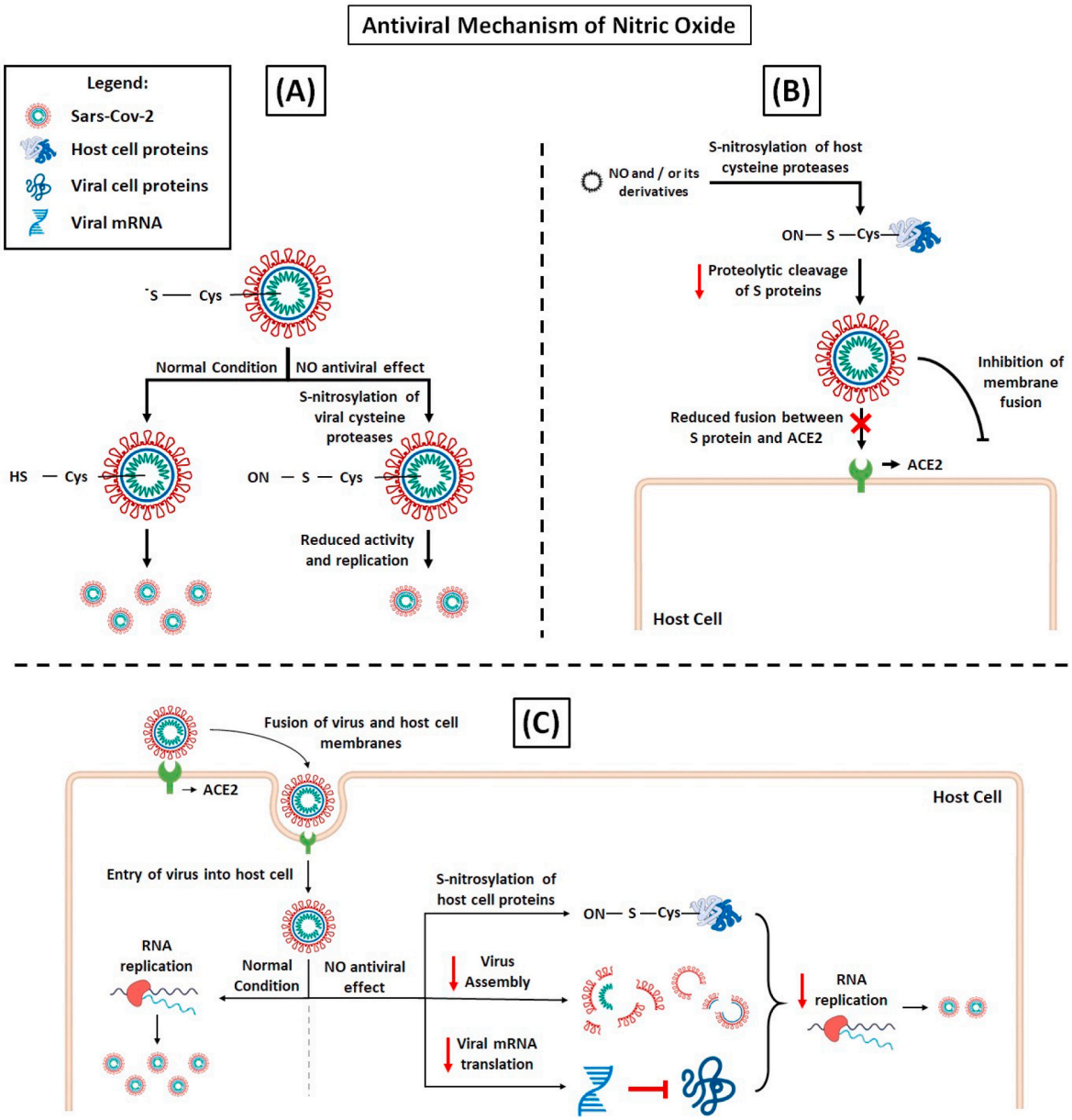


**Fig. 2.** NO antiviral mechanisms, hypothesis of action on SARS-CoV-2 replication. **(A)** Acting on viral proteases. The processing of the polyprotein region is a point of posttranslational control that is essential for virus replication. SARS-CoV-2 processes the polyproteins using two cysteine proteases, the papain-like protease ($PL^{pro}$) or the chymotrypsin-like protease ($M^{pro}$). S-nitrosylation of specific Cys residue reduces the activity of these proteases inhibiting SARS-CoV-2 replication [106,107]. **(B)** Acting on host cell proteins. The complete intracellular life cycle of SARS-CoV-2 relies on interactions with host molecules. The proteolytic cleavage of S proteins by serine protease TMPRSS2 and cysteine proteases cathepsin B (CatB) and CatL is essential for the virus fusion. Thus, the inhibition CatB and CatL by S-nitrosylation could prevent SARS-CoV-2 entry into cell. **(C)** Furthermore, NO-mediated S-nitrosylation of cysteine-containing proteins may prevent virus molecular interactions critical for RNA replication, virus assembly and translation of viral mRNAs, abrogating SARS-CoV-2 cell cycle [108,109].

tropism of the virus for ACE2 receptors, decrease the bioavailability of NO and trigger venous thromboembolism and disruption of natural antithrombotic state [76]. Also, eNOS uncoupling due to low L-arginine levels, impairs NO production or bioavailability in ARDS patients, which induces vasoconstriction and can lead to arterial and venous thrombosis [77]. On the other hand, under physiological conditions, eNOS-produced NO in the intact endothelium is released and then the NO/cGMP/PKG signaling pathway induces vasodilatation, inhibits platelet adhesion and aggregation and prevents smooth muscle cell proliferation, hindering thrombus formation [77]. Nevertheless, there is no clinically available NO therapy that addresses endothelial dysfunction directly, which could prevent thrombosis in COVID-19 patients [77,78].

Several therapeutic strategies with NO-enhancing and –releasing agents have been studied to develop new antithrombotic drugs [77]; even so, NO is not under studies to prevent coagulopathy in COVID-19.

## 4. iNOS activity: friend or enemy of COVID-19?

### *4.1. Nitric oxide in immune responses against viruses*

NO is a key molecule in the regulation of immune response to pathogens [79–83]. Mainly, iNOS-synthesized NO is an important immunoregulatory mediator of the host's immune system against

infectious organisms, and acts as a toxic agent. Futhermore, NO can regulate cellular function, growth and death of immune cells, such as macrophages, neutrophils, T cells and natural killers (NK) cells [21].

Although macrophages are the main NO producers in response to pathogens [84–86], many cells express iNOS, including fibroblasts, hepatocytes [87], endothelial and epithelial cells, keratinocytes and chondrocytes [88,89] antigen-presenting cells [90] and NK cells [21,91]. iNOS expression is induced by several agents, including microbial lipopolysaccharides and cytokines [92–94]. Once expressed, unlike eNOS and nNOS, iNOS is continuously active. Therefore, iNOS provides continuous high concentrations of NO to chemically neutralize invading pathogens, and this level of synthesis is sustained for hours or days, depending on how long the enzyme is present in cells or tissue [21].

NO has several advantages as an antiviral agent, despite that, there are few studies investigating its potential therapeutic in viral infections. Firstly, it can easily pass through cell membranes to neighboring cells, as well as to viruses, not requiring a receptor [95]. Also, NO acts on a variety of viral targets, inhibiting viral replication; and cell specificity depends on its concentration, chemical reactivity, proximity of target cells and the way that target cells are designed to respond [21]. Finally, the NO effect is independent of the immune recognition of the infected cell, in contrast to antiviral lymphocytes. Such effect may be important in virus-infected cells where the major histocompatibility complex, essential for the adaptive immune system, is limited and/or sub regulated [95]. Given the highly reactive nature of NO, its antiviral effects are probably mediated by reactions with multiple cellular and viral targets which may be advantageous for host defense because it will limit the capacity of viruses to develop resistance.

Studies have shown that the NO antiviral effects are provided by NO donors [96–100] or by iNOS directly activated by cytokines [92–94]. Rimmelzwaan et al. [101] demonstrated that influenza A virus replication in MDCK cells was severely impaired by NO generating compound, S -nitroso- N -acetylpenicillamine (SNAP). Reduction of infected cells and virus production proved to correlate with reduction of viral protein activity and viral RNA synthesis (Fig. 2A), indicating that NO affects an early stage in the replication cycle of influenza viruses. This same group hypothesizes that iNOS-synthesized NO in airways epithelial cells, induced by cytokines synthesized after virus infection [102,103], provides an antiviral effect in these cells. Likewise, exposure to NO demonstrated dose-dependent antiviral effects in cells infected with influenza A, B and H1N1 [104]. Additionally, it has been reported that peroxynitrite, an intermediate product formed by the reaction of NO with superoxide, inhibits the entry of RNA of some viruses into host cells [105].

With the new CoV global outbreak, the search for effective antivirals against SARS-CoV-2 is an important undertaking. Interestingly, inhibition of SARS-CoV replication cycle in vitro was reported with NO donors by two distinct mechanisms, by affecting spike (S) protein and ACE2 fusion (Fig. 2B) or reduction of viral RNA load in early stages of replication (Fig. 2C) [82]. Although its antiviral effects against SARS-CoV-2 is not yet elucidated, evidence shows that NO has a great potential. We hypothesize that metabolic enhancement of NO production and NO bioavailability through complex interventions can partially reverse deleterious physiological conditions associated with viral infection and unregulated pro-inflammatory processes.

### 4.2. NO in lung injuries: pro-inflammatory effect

Although NO plays a protective role in viral infection, it can also contribute to immunopathology of COVID-19. NO generation is a tightly regulated process; the pathophysiological conditions that deregulate it lead to reactive oxygen species (ROS) generation [25,110,111]. Excessive ROS produced by the endothelium and epithelium, as well as by leukocytes, play an important role in ARDS progression and lung damage. ROS positively regulate the expression of proinflammatory cytokines and adhesion molecules, causing endothelial and epithelial dysfunctions, along with increasing oxidative stress in pulmonary duct tissues and airways, further altering the inflammatory state [25,112,113]. Throughout ARDS process, lung cells release large amounts of inflammatory factors that increase iNOS synthesis in alveolar macrophages, neutrophils and bronchial epithelium, providing abundant amounts of NO for release into lung tissues [114,115]. Moreover, airway stress can induce bronchial obstruction and worsen inflammation in ARDS patients, further inducing lung tissues to produce NO [116].

Overproduction of NO leads to deleterious cell components damage when reacting with superoxide, and favors the formation of peroxynitrite that can nitrate and oxidize proteins, lipids and nucleotides [117]. In case of increased plasma NO levels, the reaction between NO and superoxide to form peroxynitrite becomes very fast, where the production rate is about three times higher than the rate of superoxide decomposition by superoxide dismutase [118]. Excessive peroxynitrite formation can lead to inhibition of mitochondrial respiration, protein dysfunction, depletion of cellular energy, damage to cell membranes and DNA [117], in addition to contributing to resistance to anti-inflammatory drugs [119]. Although reactive nitrogen species (RNS) are more widely cited, both ROS and RNS are capable of interact with proteins, DNA or lipids by oxidation or nitrosation [120].

NO-mediated oxidative stress is an important factor in the pathogenesis of lung injury. High levels of NO, represented by the increase of its stable metabolites, nitrate and nitrite, can intensify lipid peroxidation, cause necrosis and denaturation of pulmonary epithelial cells, aggravate inflammation and induce the onset of ARDS [121]. A clinical study reported high concentrations of the NO, nitrate and nitrite metabolites in the bronchoalveolar lavage, not only in ARDS patients, but also in patients at risk for ARDS, suggesting that the oxidative stress detected at the beginning of ARDS begins when patients are at risk, before the clinically defined syndrome is recognized [122].

## 5. Conclusion

As discussed in the present article, disruption of NO physiology is closely related to the development of ARDS in COVID-19 patients. Nevertheless, NO production pathways are affected in a different manner. eNOS-derived NO production is compromised, inducing alterations throughout the body, especially lung parenchyma and vessel barrier, in fact, conditions that cause changes at the epithelium increase risk for severe illness, reinforcing the important role of damaged endothelial cells in the development of severe COVID-19. On the other hand, NO production by iNOS is increased in effort to fight the virus; however, this pro-inflammatory state can cause a deleterious effect, leading to lung injury. Inhaled NO has been used in ARDS patients in the attempt to mitigate pulmonary physiological alterations caused by eNOS uncoupling, but the transitory effects and possible oxidative toxic damage may weaken the use of this therapy. Here, we propose the investigation of therapies that promote NO production in a metabolic manner. Molecules that positively modulate the activity of ASS, a key enzyme in arginine metabolism, would increase arginine production, leading to eNOS recoupling and increasing NO metabolite production [66]. On the contrary, specific iNOS inhibitors should attenuate ARDS intrapulmonary pathologies [123]. In conclusion, therapeutic approaches that modulate NO metabolism should be considered for the prevention or treatment of severe cases of COVID-19.

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