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Review

Insights on the mechanisms of action of ozone in the medical therapy against COVID-19

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ABSTRACT

An increasing amount of reports in the literature is showing that medical ozone (O_3) is used, with encouraging results, in treating COVID-19 patients, optimizing pain and symptoms relief, respiratory parameters, inflammatory and coagulation markers and the overall health status, so reducing significantly how much time patients underwent hospitalization and intensive care. To date, aside from mechanisms taking into account the ability of O_3 to activate a rapid oxidative stress response, by up-regulating antioxidant and scavenging enzymes, no sound hypothesis was addressed to attempt a synopsis of how O_3 should act on COVID-19. The knowledge on how O_3 works on inflammation and thrombosis mechanisms is of the utmost importance to make physicians endowed with new guns against SARS-CoV2 pandemic. This review tries to address this issue, so to expand the debate in the scientific community.

1. Introduction

Ozone (O_3) is an unstable molecule, a chemical allotrope of O_2, which was recently used in a standardized mixture with oxygen to successfully treat COVID-19 alongside with usual anti-inflammation pharmacology [1-11], so representing a possible encouraging approach to address COVID-19 [12]. To date, the molecular mechanisms with which O_3 is able to act against COVID-19 are yet far to be fully elucidated, though several attempts on how O_3 might work in biological systems were recently reported [13-19]. Although O_3 can easily remove SARS-CoV2 from inert surfaces, an evidence assessing its well known virucidal potential [20-23], the activity of O_3 in the human organism is radically different respect to the gaseous O_3 used for environmental disinfection. As a matter of fact, despite some reported evidence showing the direct pro-oxidant use of O_3 against microbial infections [24], medical O_3 usually hampers virus spreading via O_3-generated mediators, such as lipid-derivatives (aldehydes and oxysterols), which are potent SARS-CoV2 inhibitors [25-27]. More generally, when talking about O_3 in medicine, it should be mandatory to distinguish a "pollutant airborne O_3", usually toxic and which may directly interacts with airway

Abbreviations: Bach1, BTB Domain and CNC homolog 1; Bach2, BTB Domain and CNC homolog 2; COVID-19, coronavirus disease 2019; Cys, cysteine; EC_{50}, concentration giving half-maximal response (effect concentration 50%); eNOS, endothelial nitric oxide synthase; ERK, extracellular signal regulated kinase; FAS/TNFR, recettori di morte programmata cellulare; GSH-Px, glutathione peroxidase; iNOS, inducible nitric oxide synthase; HMOX1, heme oxygenase 1; HP, haptoglobin; HUVEC, human umbilical chord endothelial cells; IRAK-1, interleukin-*1* receptor-associated kinase 1,; LCN2, lipocalin 2; LGP2, Laboratory of Genetics and Physiology 2; LPS, lipopolysaccharide, MDA, malonyldialdehyde; MDA5, melanoma differentiation-associated protein 5; NADPH, nicotinammide dinucleotide phosphate; Neh2, Nrf2-ECH-like homology 2; NF-E2, Nrf2-ECH family; Nrf2, nuclear factor erythroid 2-related factor 2; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NO, nitric oxide; NO_2, nitrogen dioxide; PaO_2/FiO_2, arterial oxygen pressure on inspired fraction of oxygen; p38MAPK, p38 mitogen activated protein kinase; RIG-1, retinoic acid-inducible gene 1; RTA-408, omaveloxolone; S100A8, S100 Calcium Binding Protein A8; SAE, SUMO activating enzyme; SARS-CoV2, severe and acute respiratory sindrome-coronavirus 2019; SUMO, small ubiquitin-like modifier; SOD, superoxide dismutase; THP-1, a human leukemia monocytic cell line; TNF-α, tumor necrosis factor alpha; TRAP-1, TNF Receptor Associated Protein 1); VAV-1, vav guanine nucleotide exchange factor 1.

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epithelia, from a "medical gaseous O_3", which is usually administered in a balanced O_2/O_3 mixture via autohemotherapy, or rectal insufflation or, in laboratory animals, also as peritoneal injection [28].

The long history of O_3 in medicine, used to treat a wide plethora of illnesses, dates back to Dr Walls' in 1915 and has gradually faded off its pioneering empirical hallmark to come to an excellent, straightforward expertise in employing O_3 successfully, even in chronic and degenerative disorders [29-34]. Very recently, Franzini et al.'s, using O_2/O_3 autohemotherapy (O_2-O_3-AHT), succeeded in significantly reducing hospitalization in intensive care units (ICUs) of 50 male patients (mean age 75 yrs ± 11.4 SD), from a median of 22.13 ± 3.44 days to 13.45 ± 2.33 days (41% reduction), an effect probably due to the concurrent effect of O_3 on the usually recommended therapy protocol for COVID-19 [3]. Many further research groups are confirming these results, though with different protocols. Tables 1A and 1B summarizes the more recent evidence in using O_3 to treat COVID-19. Despite the several efforts to assess the role of O_3 in reducing inflammation and pro-thrombotic mechanisms, the paucity of clinical papers exerts a relevant impact on the effect size when the major parameters reported in those papers are gathered for *meta*-analytic investigation. Tables 1A and 1B reports the statistics of the effect power for IL-6 and CRP (as main markers of inflammation) and D-dimer (as a major marker of thrombotic events), resulting that inflammation is reduced by 80% ($p < 0.05$), whereas thrombosis is reduced by 50% ($p > 0.05$), yet these data need to be further assessed. This review has the objective to gather the bulk of evidence regarding the ability of O_3 to reduce inflammation and pro-thrombotic events, throughout the literature on the latest 30 years, in order to give insights about how ozone can exert its positive action on COVID-19 patients, currently emerging in the clinical reports.

2. Brief focus onto the biology of ozone

Clinical success suggests that the role of O_3 in human physiology may be much more important than expected, as O_3, or at least its major oxidative byproducts, are physiologically present in the organism. As a matter of fact, past reports addressed the intriguing hypothesis that natural immune cells, such as neutrophils, may produce biological ozone [35-37], though controversial opinions were also raised about [38,39]. Yet, recent studies have reported that the exposition of amino acids or antibodies to singlet oxygen may form biological ozone [40]. Oxygen radicals and O_3 are therefore frequent byproducts of the many oxidative processes involving bio-molecules. This evidence should suggest that O_3 may work more frequently as an inside biological molecule, rather than a chemical xenobiotic, probably acting as a chemical switcher of the complex interplay made by oxidative stress response, immunity and even vascular physiology. As we are going to address further on, O_3 is not only able to modulate oxidative stress, which is considered a leading causative factor in COVID-19 [41], but also to work as a master regulator of the complex cross talk between oxidative stress and inflammation, including blood coagulation and endothelial physiology. Recent evidence reported that during COVID-19, genes involved in the stress response, such as TRAP-1 (expressing heat shock protein 75, hsp75) and NOX (expressing NADPH oxidases) are deregulated, alongside with SAE (encoding for protein SUMOylation), VAV1 (implicated in platelet functions and blood coagulation) [42] and the expression of several cathepsin proteases [43]. The pathogenetic scenery where O_3 should work may be summarized as follows. SARS-CoV2 induces a robust type I/III interferon response via the activation of the innate immunity, inflammation and subsequently adaptive immunity, but the dysregulation of the rennin/angiotensin system caused by disturbing the angiotensin-converting enzyme 2 (ACE2) signaling, finally leads to oxidative stress, then tissue damage and a widespread triggering of the coagulation cascade causing disseminated intravascular coagulation (DIC) and finally thrombosis [44]. Oxidative stress, inflammation and coagulation disorders are closely intertwined in COVID-19 pathogenesis and these may represent fundamental targets for O_3-mediated therapy.

However, from a pharmacological point of view, O_3 is widely considered a simple pleiotropic molecule. It should be able, therefore, to fundamentally target the complex cell machinery involved in responding to the oxidative stress and then to act in a rather aspecific way on immune modulation, yet depending on the different O_3 exposure, dosages and experimental conditions [45-48]. As previously introduced,

Table 1A
Recent clinical studies and in progress trials on the application of ozone therapy (O_2-O_3-AHT) against COVID-19.

Study	Sampling	Ozone method	Main results		References
Case study	50 male patients COVID-19 positive in ITUs, mean age 75	200 ml 45 μg/ml O_2-O_3 MAHT (SIOOT protocol)	↓	IL-6, inflammatory markers, LDH, CRP, D-dimer	[3]
			↑	$SatO_2$%, PaO_2/FiO_2, time of hospitalization	
RCT	60 patients, aged 30–60, both sexes, with mild to moderate COVID-19	150 ml 40 μg/ml O_3 twice daily rectal insufflation plus 5 ml 25 μg/ml O_2-O_3 mAHT	↑	Cases of negative SARS-CoV2 RT-PCR (100% on day 10 following treatment), relieved breathlessness, SpO_2	[4]
			↓	CRP, LDH., ferritin, hospitalization times	
Prospective case control study	18 patients, (9 controls + 9 treated), COVID-19 infected and hospitalized	O_2-O_3 MAHT 200 ml blood O_2/O_3 misture and O_3 40 μg/mL	↓	Days of hospitalization. Ferritin, CRP, D-dimer, LDH	[5]
2 case reports	Patient 1 male 53 yrs COVID-19 with pneumonia	O_2-O_3 MAHT O3 conc not specified	↓	CRP, LDH	[7]
			↑	Leukocyte, PaO_2, $SatO_2$% ,	
	Patient 2 male 66 yrs COVID-19 severe pneumonia		↓	CRP, LDH	
			↑	PaO_2, $SatO_2$%	
Case control study	14 + 14 (treated/control) patients positive for COVID-19	150 ml at a concentration of 35 μg/mL for 5 to 10 days	↑	Oxygen saturation %, lymphocytes %	[10]
			↓	Fibrinogen, D-dimer, LDH, CRP, IL-6, Taylor scale	
Current clinical trials and trial plannings					
Interventional RCT	208 participants COVID-19	100–200 ml of blood with O_3 40 μg / mL 200 ml every 12 h during 5 days.	↑	Rate of improvements at 14 days (1 end point) Clinical condition (1 end point)	NCT04370223
			↓	Mortality at 28 days (2 end point)	
Interventional RCT	50 participants COVID-19 crossover assignment	200 ml O_3 40 μg/mL of medical O_2/O_3 in 200 ml	↑	COVID-19 clinical scale (1 end point) 3 weeks	NCT04359303
			↓	Mortality at 21 days (2 end point)	
Observational cohort prospective	25 patients COVID-19 with pneumonia	200 ml O_3 40 μg/mL of medical O_2/O_3 in 200 ml	↑	Clinical state (1 end point)	NCT04789395
			↓	Mortality (2 end point)	

Table 1B
Major investigated markers in the recent clinical studies on O_3 in COVID-19.

Study		Method ozone	IL-6	CRP	D-dimer	LDH	PaO2/FiO2	SatO2%	Days	References
1	4 case reports	Rectal 100 ml of rectal ozone, at a concentration of 35 μg/mL for 5 to 10 days,	48,7 8,2 ↓ 83.16%	1,1 0,85 ↓ 22.73%	1965 585,5 ↓ 70,20%	253 210 ↓ 16,99%		89 97,5 ↑ 9,55%		[224]
2	Case control study on 14 patients	8 sessions (1 session/day) of intra-rectal ozone (150 ml volume, 35 μg/mL concentration [5.25mg total dose])			3240 1343 ↓58,55%			92.96 94.3 ↑ 1,44%	35.67 28.58 ↓19.88%	[10]
3	Case study	50 O2-O3-AHT 45 ug	660 90 ↓ 86,36%	15 5 ↓ 66,67%	1250 500 ↓ 60.00%	350 290 ↓ 17.14%		80 95 ↑ 18.75%		[3]
4	RCT	60 40 ug 150 2 v al di con mini emo		0.98 0.85 ↓ 5.55%		944.0 752.6 ↓ 20.27%		96.4 97 ↑ 0.62%		[4]
5	Prospective case control study	18 patients 200 ml 40 ug			943 389 ↓58.75%	4,3 2,9 ↓ 32.56%				[5]
7	4 case report	100 ml 40 ug	166,4 10,1 ↓ 93.93%	300 10,2 ↓ 96.6%						[225]
8	Clinical trial	200 ml 40 ug 60 pat 30 +30	44 33 ↓ 25.00%	76,4 67,6 ↓ 11.51%						[8]
9	Case control study	28 (14 +14) 30 ug	104,5 44,57 ↓ 57.34%	164,54 46,40 ↓ 71.80%	1187 914,8 ↓ 22.93%					[9]

↓ reduction respect to control or before treatment; ↑ increase respect to control or before treatment.
Statistical power for each evaluated parameter. 1) effect of O_3 on inflammation = reduction of 80% (power 0.80, $p = 0.04878$); Effect of O_3 on coagulation reduction of 56.53% (power 0.50, $p = 0.055556$). Effect size: Hedges (SMD) fixed effect model = 1.54 ; $CI_{95} = [0.963,2.122]$ z score = 5.215p < 0.0001 $I^2 = 97.899999999999999\%$, $Chi^2 = 193.4$, random effect model = 9.07; random effect model = 9.07; $CI_{95} = [3.756,14.376]$, z score = 3.346, $p = 0.000819$ $I^2 = 97.899999999999999\%$, $Tau^2 = 32.7$
The evaluation shows a high heterogeneity and a large effect size (Rosenberg, M. S. (2005). The file-drawer problem revisited: a general weighted method for calculating fail-safe numbers in meta-analysis. Evolution, 59(2), 464–468.
LEGEND CRP: C-reactive protein; ITUs : intensive therapy units; O_2-O_3-MAHT: major oxygen-ozone autohemotherapy; O_2-O_3-mAHT: minor oxygen-ozone autohemotherapy SIOOT: Italian Society of Oxygen Ozone Therapy; SatO2% : percentage of oxygen saturation; PaO_2/FiO_2 the ratio of arterial oxygen partial pressure (PaO_2 in mmHg) to fractional inspired oxygen (FiO_2 expressed as a fraction, not a percentage), SpO_2: oxygen saturation.

medical O_3 usually uses this gas in an oxygen-ozone mixture injected via autologous hemotransfusion and should not be mismatched with airborne O_3 coming from environmental pollution, which may be toxic for organisms when associated with long term exposure and presence of further pollutants such as NO_2 and particulate matter [49,50]. Ozone is toxic if directly inhaled, even in relatively moderate concentration [51], so this way of assumption is never considered in a therapy approach. Despite it is a chemical toxicant, O_3 exerts its beneficial action depending on its dosage, therapy protocol, biological microenvironment and genetic or epigenetic factors, which appear to be fundamental in the development and pathogenesis of COVID-19 [52]. This beneficial effect is mainly exerted by blood-derived byproducts. On airway and lung epithelia, gaseous O_3 is often noxious because it damages lung surfactant protein B (SPB), leading to respiratory distress [53]. SPB is a member of a group of proteins, present exclusively in the lung epithelia, with antimicrobial activity (SPA and SPD) or which interacts with phospholipids,

such as di-palmitoyl phosphatidylcholine (DPPC) to ensure a surface-active air–water film and prevent lung collapse [54].

As an allotrope of oxygen, O_3 is particularly instable in aqueous solutions, such as plasma of circulating blood [55,56], probably because O_3, or its major oxidative byproduct —OH* radical, are rapidly quenched by anti-oxidants such as cysteinyl-groups in proteins, uric acid, ascorbic acid, reduced glutathione (GSH) or even albumin [57]. When injected into the blood, O_3 may react with poly-unsatured fatty acids (PUFA), generating hydrogen peroxide [58], which is produced also by the O_3 interaction with molecules containing aldehyde groups, therefore forming lipid oxidation products (LOPs). One of these products is 4-hydroxynonenal (4-HNE) [58], which is a powerful bioactive molecule, as demonstrated by past reports showing that 4-HNE mimics O_3 in inducing a modulation of the macrophage function *ex vivo* [59]. O_3-generated byproducts might work as signaling molecules in those mechanisms demonstrating the immuno-modulatory effect of O_3. As O_3 may interact at least theoretically with any organic molecule, its ability in generating bioactive mediators, such as LOPs, allows this molecule to finely regulate the complex interplay between oxidative stress and inflammation, so targeting many actors involved in the plasma-endothelial cross talk of the vascular system, probably promoting anti-thrombotic effects even on COVID-19 patients [1-3].

3. O_3 in the interplay oxidative stress-inflammation

3.1. Targeting the pathway Nrf2/Keap1/ARE and the NF-κB signaling

Impairment in the anti-oxidant/inflammatory axis, exerted by the interplay Nrf2/NF-κB, may be a leading cause of severe exacerbations in COVID-19 [60]. O_3, by generating active functional species, should be considered a promising tool for treating patients with COVID-19, even upon an agreed pharmacological protocol [61]. Yet, this perspective needs to be further assessed by further elucidating O_3 mechanisms of action occurring to counteract COVID-19 pathogenesis.

Cuadrado et al., recently wondered if the activation of the nuclear factor erythroid-derived 2-like 2 (Nrf2) may be a successful strategy against SARS-CoV2 [62]. Being a gene transcription factor, Nrf2 controls the stress-mediated expression of a wide array of the antioxidant response element (ARE)-dependent genes, principally involved in the scavenging of the reactive oxygen (ROS) and nitrogen (RNS) species [63]. The activity of Nrf2 is switched on to reduce also an excess of oxidative stressors, whereas activators of Nrf2 can be used to pharmacologically respond to the oxidative stress. For example, the synthetic Nrf2 activator, RTA-408, suppresses, by activating Nrf2, the excess ROS production following an injury, by inhibiting also γδTh17 cells [64]. Nrf2 works therefore as a major switcher in the anti-oxidant response following an oxidative injury. Oxidative stress provides a fundamental contribution in the development and exacerbation of COVID-19 [41]. This encourages researchers to seek for novel therapeutic suggestions, targeting Nrf2 to treat COVID-19 [65] Nrf2 is linked in the cytoplasm with Kelch like-ECH-associated protein 1 (Keap1) and with Cullin-3, which can degrade Nrf2 via ubiquitination, as Cullin-3 ubiquinates Nrf2 and Keap-1 promotes this reaction by binding to the Nrf2 conserved amino-terminal Neh2 domain [66]. Mammals are endowed with several hundreds of ARE-driven genes. The genetic region containing the sequence 5′-A$^{/G}$TGAC$^{/T}$nnnGCA$^{/G}$-3′ is the core box of an ARE-regulating *cis*-acting element [67]. Many oxidative stress-derived molecules, including O_3 and its oxidized mediators, such as hydroxyl radical (OH^-), carbon mono-oxyde (CO), nitric oxide (NO), peroxynitrite (ONOO—), peroxyntrous acid (ONOOH) and hypochlorite (HOCl), can directly activate ARE-dependent gene expression [68]. Moreover, Nrf2 is a component of the “cap ‘n collar” (CNC) family, collecting at least six factors in mammals, i.e. p45, Bach1, Bach2, Nrf1, Nrf2 and Nrf3, representing the NF-E2 subfamily, which forms active dimers able to enhance or inhibit ARE-dependent gene expression [68].

Recent studies have demonstrated that O_3 activates Nrf2 in a dose-dependent manner [69,70]. Actually, several reports have shown the ability of medical O_3 to reduce oxidative stress [71-73] but even to modulate the Nrf2/NF-κB interplay, probably affecting the IL-6/IL-1β rate of expression in COVID-19 [72,73]. Furthermore, NF-κB interacts, via p65, with Keap1, so repressing the Nrf2-ARE pathway [74]. O_3 activates Nrf2 and inhibits the NF-κB pathway [69,75], therefore showing anti-oxidant and anti-inflammatory properties [76]. This ability is possessed also by ozonized low density lipoproteins (ozLDLs), which can inhibit NF-κB via the down-regulation of the IRAK-1 associated signaling [77]. Therefore, medical O_3 in the plasma can generate ozLDLs, which induce decrease in IκBα proteolysis, reduction in κB-dependent gene transcription and the phosphorylation and proteolysis of the IL-1 receptor associated kinase 1 (IRAK-1), so triggering an anti-inflammatory pathway [77]. Chemical interaction of O_3 with peripheral blood, which should occur during its medical use via the O_2-O_3-AHT [1-3], generates a huge deal of biochemical mediators, which probably work on the Nrf2/NF-κB interplay via a hormetic dose-response mechanism [78].

The concept of “hormesis”, firstly reported by Calabrese and Baldwin in 1998 [79], which has been recently associated with the concept of “mild stress” or “eustress” [69], was introduced for O_3 by Bocci and colleagues, to highlight the beneficial effect of relatively low doses (or low exposure) of O_3, which usually, at high doses, is a pro-oxidant and potentially toxic molecule [78]. Interestingly, likewise many xenobiotics inducing benefits by a hormetic mechanism [78-81], O_3 interacts with aryl-hydrocarbon receptors (Ahr), controlling lung inflammation by modulating the IL-22-mediated signaling [82], a way used also by plant derived phyto-chemicals, to induce an anti-inflammatory response [83]. According to some authors, the hypothesis by which O_3 should induce the Nrf2-pathway activation, may involve the onset of a mild oxidative stress, able to elicit the expression of the antioxidant endowment of the cell, without causing stress-related injury [84,85]. Oxidative stress response is an early mechanism modulating immunity and actually the Nrf2/Keap1/ARE pathway is of major importance in inflammation [86,87], particularly in COVID-19 [65].

As a matter of fact, recent evidence reported that SARS-CoV2 dampens the activity of Nrf2 signaling, as the Nrf2 pathway-mediated expression of the antioxidant genes is suppressed in biopsies from patients with COVID-19 [88]. Recent reports have shown that the transcriptome analysis of lung biopsies from patients with COVID-19 showed an enrichment in the expression of genes associated with inflammation, such as Toll-like receptors (TLRs) and the RIG-I like receptors RIG-I, MDA-5 and LGP2, whereas a strong reduction in the genes associated with Nrf2 was observed [88]. The activation of the Nrf2-mediated signaling appears therefore pharmacologically strategic in the COVID-19 treatment. Furthermore, cells produce molecules able to trigger the Nrf2-mediated pathway, such as fumarate and itaconate [88]. While fumarate is a common citrate and urea cycle intermediate, itaconate is produced by the aconitate decarboxylase I in macrophage mitochondria, usually upon inflammatory or xenobiotic stimuli. Following Keap 1 alkylation, itaconate induces an Nrf2-mediated response [89]. Furthermore, during a chronic lung disease a metabolic reprogramming of airway macrophages does occur, as these innate immune cells use the ROS signaling to produce itaconate, which fundamentally is able to dampen bacteria infection, such as *P aeruginosa,* by inhibiting the microbial isocitrate lyase in the shunt of glycoxylate [90,91]. Itaconate in airway macrophages is a leading anti-microbial molecule and its production is activated by ROS signaling, probably by molecules able to trigger an oxidative stress response such as O_3 and its mediators [92]. Many of these mediators are produced by O_3 in the blood.

3.2. Anti-inflammatory property of O_3 via oxidized mediators and research evidence

Macrophages highly express two fundamental receptors, i.e. the sterol receptor element binding protein (SREBP) and the liver X receptor alpha (LXRα), which regulate cell response and cytokine release [93,94].

The role of SREBP is fundamental because is increased, via the NF-κB signaling, by an inflammasome-mediated pathway in M1-pro inflammatory macrophages, whereas the anti-inflammatory M2 phenotype is activated by a LXRα-mediated pathway [92]. O_3 in the blood is able to produce a great deal of lipid oxidized products (LOPs), and O_3 itself may have a major role in modulating the response of innate immune cells and the macrophage M1/M2 phenotype switching [95]. Cholesterol may be oxidized by O_3 and its oxidant radicals in the blood, forming products generally known as oxysterols, which can interact with LXRα [96]. Oxysterols include a wide family of oxidized cholesterol byproducts, which exert an immuno-modulatory role [97]. In the lung, O_3 derived oxysterols exert primarily a pro-inflammatory activity as quite exclusively interacting with the SREBP-mediated pro-inflammatory signaling in airway type II cells, due to the presence of surfactant proteins [98,99]. In the blood, O_3 can generate several lipid-derived mediators, besides to oxygen and nitrogen-derived radical species, even from polyunsaturated fatty acids (PUFAs), including oxysterols interacting with LXRα [100-102]. According to Bocci, the "therapeutic window" for O_3 might range from 0.21 μmol/ml (10 μg/ml O_3 for each ml of blood) to 1.68 μmol/ml (80 μg/ml O_3 for each ml of blood), as in this dosage range the anti-oxidant system is able to neutralize O_3 and to maintain its biological benefit, whereas higher doses are undoubtedly toxic, following the U-shaped paradoxical pharmacology of hormesis [103]. Besides to ROS, O_3 may produce reactive electrophilic species (RES), such as α,β-unsaturated aldheydes from PUFA and interestingly, at least from a functional point of view, itaconate too is a RES, being able to activate macrophages in responding to stress via mito-hormesis, a mechanism suggested also for O_3 [78,104,105]. The oxidation of lipids such as arachidonic and linoleic acids by O_3 may form α,β-unsaturated hydroxyalkenals [78,106], such as the same 4-hydroxy-2-nonenal (4-HNE), which has a leading role in the anti-oxidative response via Nrf2 even in human lung cells [107,108]. Actually, the relative short-time contact of O_3 with blood, during an O_2-O_3-AHT, allows O_3 to react with ω-3 PUFA, forming hydroxyl-hexaenal (HHE) or with ω-6 PUFAs forming 4-HNE [109]. This latter, enabling chemical adducts with Cys-34 residue in the albumin, may trigger, in picomolar concentrations, an oxidative Nrf2-mediated stress response [109]. Literature about 4-HNE describes this byproduct of lipid peroxidation as an inducer of oxidative stress, then involved in several oxidant-induced disorders, despite the evidence that, at low doses, 4-HNE exerts a beneficial, anti-oxidant and cytoprotective action via the induction of the thioredoxin reductase 1 from Nrf2 activation [110]. The anti-oxidant and anti-inflammatory role of 4-HNE has been recently reviewed [107]. Low doses of 4-HNE (5–10 μml/L) enhances the expression of heme oxygenase-1 (HO-1), contributing in protective endothelia and vascular physiology [111].

The biological activity of O_3 is mainly mediated by cholesterol-derived oxysterols, electrophiles such as α,β-unsaturated aldheydes from PUFA and modified cysteinyl (Cys) residues in proteins. None of these byproducts are beneficial *per se*, being, as other oxidized end products, toxic at high concentrations. Yet, they may trigger, as signaling molecules, the expression of cytoprotective and survival genes [112]. RES such as 4-hydroxy-2-hexenal, 4-HNE, 15d-$\Delta^{12,14}$-PGJ2, induce a cell adaptive response as they can disrupt the Nrf2-Keap1 complex via the modification of Cys273 and Cys288 of the at least 25 Cys residues in Keap-1, then activating Nrf2 [113]. Actually, the ability of O_3 to interact with cysteinyl residues, may tune the activity of strategic Cys-residues in the Keap1 function. Three major cysteine sensors were reported in the Keap1 involvement in the stress response, namely Cys151, Cys273, and Cys288, which work as sensing stressors to activate the anti-oxidant Nrf2-mediated machinery [114,115]. The chemical modification exerted by O_3 on Keap-1 crucial Cys residues in their thiol groups, should inhibit the Nrf2 shut-down by Keap1, so prolonging the oxidative stress response by O_3 [109]. The interaction of O_3 with blood, forming important metabolites able to trigger an anti-oxidant response at low doses, should be a crucial issue to be further expanded and investigated in pharmacology [58].

In rheumatoid arthritis purified synovial fibroblasts, 3–5% v/v of gaseous O_3 reduced cell expression of TNF-α, IL-1β and IL-6 [116], pro-inflammatory cytokines actively participating in COVID-19 pathogenesis [117]. O_3-derived metabolites such as 4-HNE, are able to inhibit IL-6 production in liver macrophages by acting on NF-κB, i.e. preventing its activation and suppressing the phosphorylation of IκBα [118], and noteworthy 4-HNE inhibits both TNF-α and IL-1β expression in the human monocytic cell line THP-1 in response to LPS [119].

3.3. *Anti-inflammatory and anti-thrombogenic property of O_3: Role of HO-1, HIF-1α*

As described before, O_3 may activate an anti-oxidant response via the Nrf2/Keap-1/ARE pathway either eliciting a ROS-mediated signaling by lipid oxidized products, such as oxysterols and α,β-unsaturated aldheydes from PUFA or by eliciting other mediators such as heme oxygenase-1 (HO-1) [120]. Both O_3 and 4-HNE induce the production of HO-1, connecting the Nrf2/NF-κB cross talk with endothelia physiology and coagulation [111,120]. The oxidative stress has been suggested as a leading issue in the COVID-19 pathogenesis [121,122], therefore any pharmacological strategy to dampen oxidative stress in SARS-CoV2 infected subjects is of the utmost importance. In this context, some authors have suggested that targeting HO-1 may be a promising step in controlling SARS-CoV2 infection and addressing a successful COVID-19 treatment [123]. Heme oxygenases, i.e. heme oxygenase 1 (HO-1) and heme oxygenase 2 (HO-2), not only degrade physiologic heme then releasing CO, biliverdin and iron, but act as oxygen sensors during hypoxia [124]. Actually, one of the leading causes of COVID-19 exacerbation, often associated with co-morbidities such as obesity, is hypoxia [125]. Obesity, which is a major comorbidity in COVID-19, may enhance the production of the hypoxia-inducible factor-1α (HIF-1α), shifting an existing cytokine storm to a fulminant event [125]. As HO-1 is one of the genes expressed by the activation of the Nrf2/Keap1/ARE pathway and being a major tuner of blood O_2 level, its induction by O_3 and O_3-derivatives may be particularly crucial for successfully treating COVID-19 patients. Mammalian cells must regulate their oxygen levels to the proper homeostatic balance. In this sense, HIF-1α is a molecular oxygen sensor, a subunit of the heterodimeric gene transcription factor HIF-1 together with HIF-1β, and encompasses the analogs HIF-2α and HIF-3α [126]. As like as Nrf2, also HIF-1α has a DNA binding motif, called hypoxia response element (HRE) [127]. The role of O_3 towards HIF-1α has been recently addressed in experimental animals with diabetic nephropathy, resulting in a decrease of the apoptotic signal by inhibiting the expression of caspases 1,3, and 9 and modulating the activity of HIF-1α [128]. Fundamentally O_3 seems to inhibit HIF-1α expression [129], so reducing the hypoxic stimulus. Moreover, the interplay Nrf2-NF-κB and HO-1 regulates the expression of the vascular cell adhesion molecule-1 (VCAM-1), inhibiting their expression, which is normally up-regulated in COVID-19 [130,131].

A complex interrelated functional network can be described involving the cross talk Nrf2/NF-κB in the activity of hormetic doses of O_3 and its derivatives in the blood [73]. In the blood O_3 may form ROS from water, reactive nitrogen species (RNS) from oxidized nitrogen and RES from lipoproteins, membrane lipids and other PUFA derivatives such as 15deoxy-$\Delta^{12,14}$-PGJ2 [132,133]. These byproducts trigger an anti-oxidant mechanism via the Nrf2/Keap1/ARE activation, so inhibiting the pro-inflammatory machinery led by the NF-κB pathway. Moreover, the interaction of RES with Cys residues in Keap1, reduces the proteasome-dependent degradation of Nrf2, enhancing its activated state and by inhibiting the apoptotic pathways, both the FAS/TNFR/caspase 8 signaling and the mitochondria-mediated apoptosis, induce the activation of survival genes [134]. Briefly speaking, the activation of an anti-oxidant mechanism via Nrf2 induces an inhibition of the pro-inflammatory mechanism suppressing NF-κB activation. Moreover, the activation of the NRf2/Keap1/ARE triggers the production of HO-1, which is induced by O_3 directly and inhibits platelets-dependent

thrombosis [135-138].

Fig. 1 summarizes this overview.

In addition, the oxygen saturation percentage ($SatO_2$%) as well as other lung function parameters such as the ratio arterial oxygen pressure on inspired fraction of oxygen (PaO_2/FiO_2), are fundamental markers in COVID-19 pathology, therefore mediators of oxygen homeostasis are possible targets of the medical O_3 in COVID-19 therapy. When normal and physiological levels of oxygen are present, the alpha subunit of HIF-1 is hydroxylated on specific Pro residues in the O_2-dependent degradation domain by the prolyl-hydroxylase domain containing proteins (PHDs). The hydroxylated HIF-1α form is recognized by the von Hippel-Landau protein, then targeting HIF-1α, HIF-2α and HIF-3α for ubiquitination and degradation by the 26S-proteasome [139]. With low oxygen levels HIF-1α cannot be longer degraded, due to impairment in PHDs activation, so accumulating HIF-1α, which is translocated to the nucleus where activates HRE-dependent genes, such as TNF-family death receptors inducing apoptosis [139]. In this sense, the reduction of HIF-1α level by O_3 may be explained as a counteracting action to reduce the pro-inflammatory and pro-apoptotic signal led by the HIF-1α on HREs. Recent reports have outlined a major cross-talk between HIF-1α pathway and Nrf2 signaling, suggesting that the role of O_3 in this context may be tunable and intertwined with the Nrf2/Keap1/ARE signaling [139,140]. During hypoxemic stimuli caused by COVID-19 associated pneumonia, the role of HIF-1α appears particularly intriguing, because HIF-1α up-regulates ACE-1 receptors, therefore reducing the expression of ACE-2 ones. As a balance ACE-1/ACE-2 receptors exists in

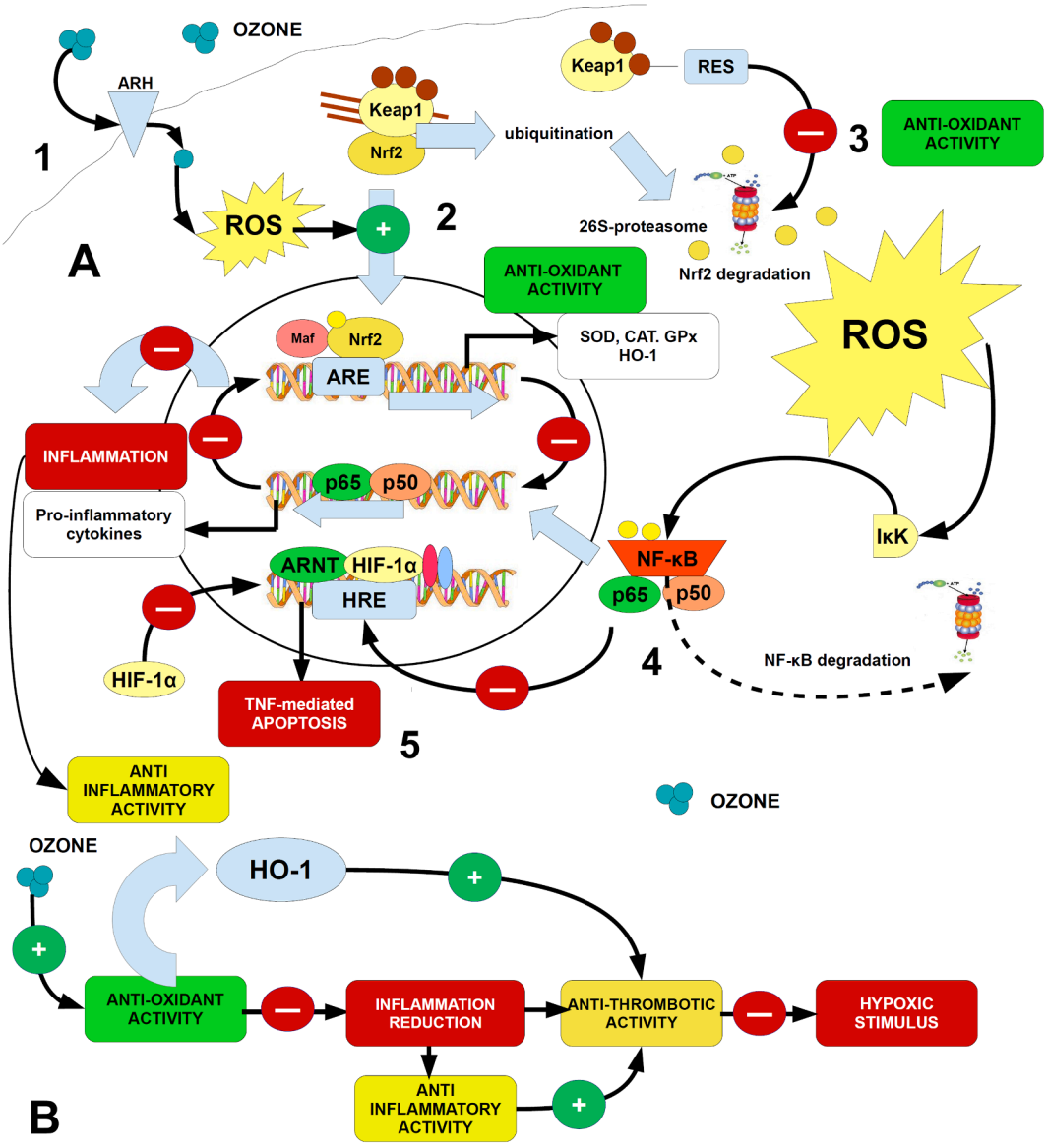

Fig. 1. Cartoon showing the major pathways targeted by O_3 and its ROS and RES mediators on COVID-19. 1) O_3 can even enter the cell via aryl-hydrocarbon receptors (AHR) and may form ROS or RES, both able to activate the Nrf2/Keap1/ARE system, inducing an anti-oxidant response. As the activation of Nrf2 blocks the NF-κB signaling, Nrf2 activation inhibits the inflammatory signal (anti-inflammatory action). 2) The anti-oxidant response is enhanced by blocking the Keap-1 mediated degradation of Nrf2; 3) The activation of Nrf2 releases HO-1, which exerts an anti-thrombotic action and moreover inhibits p65 expression and translocation into the nucleus, so suppressing the NF-κB pathway (anti-inflammatory action); 4) the HO-1 mediated anti-thrombotic action promotes the reduction of organ damage in I/R injury models, which are protected by the HO-1 stabilized HIF-1α,. Nitric oxide (NO), elicited by the anti-thrombotic action, increases the production of HO-1, so emphasizing the beneficial effect of HO-1 on vascular endothelia, dampening thrombotic mechanisms and promoting cardiovascular protection. 5) Hypoxia induces the activation of the HIF-1α pathway, which up-regulating the expression of ACE-1 receptors and subsequently down-regulating ACE2R, inhibits SARS-CoV2 spreading in the organism. HIF-1α enhances the production of HO-1. Green circles (+) = activation, red circles (-) = inhibition. Ozone is indicated by the picture with 3 full circles.

physiological conditions, HIF-1α therefore reduces SARS-COV2 spreading in the organism [141]. Stabilization of HIF-1α is considered, therefore, fundamental to dampen SARS-CoV2 infection [141] and HO-1 stabilizes HIF-1α, protecting the organism from the ischemia–reperfusion injury [142]. Moreover, HIF-1α, in mild stress conditions, promotes the expression of HO-1, so enhancing the antithrombotic and cardiovascular protective mechanisms [143]. Low oxygen promotes SARS-CoV2 replication [144]. In this sense, the role of O_3 in restoring optimal oxygen availability may be crucial also in reducing viral spread in multiple organs and tissues. In this respect past investigations have reported that O_3, when in contact with human blood, is able to elicit a remarkable up-regulation of HO-1. The percentage of increase in HO-1, was + 132% (as bilirubin, pmol/mg cell protein/1 h on HUVEC treated with 40 μg/ml O_3 in an O_2/O_3 mixture in plasma), +156% with 40 μg/ml O_3 and + 116% with 80 μg/ml O_3, showing a possible hormetic effect [120].

This should emphasize the role of O_3 in counteracting oxidative stress, inflammation and ischemic-thrombogenic processes. Actually, HO-1 has been recently considered a pharmaceutical target for COVID-19 treatment [145]. During SARS-CoV2 infection HO-1 increases in the blood alongside with heme, anemia and desaturation [146]. The evidence reporting that O_3 can activate HO-1 may represent a promising approach to treat COVID-19, for which targeting HO-1 is an issue of increasing interest [147,148]. HO-1 and Nrf2 plays a fundamental interplay in the oxidative stress response, as HO-1 is a Nrf2-regulated downstream gene [146], therefore it is tempting to speculate that the activity of O_3 on HO-1 may be indirectly tuned by a much more direct effect on the Nrf2/Keap-1/ARE pathway, as suggested above.

3.4. Anti-inflammatory property of O_3: Some recent insights on immune cells

Elucidating the action of O_3 and its derived ROS and RES on immune cells is fundamental, due the involvement of immunity in COVID-19 [149-151]. Ozone modulates the differential expression of pro-inflammatory M1 and anti-inflammatory M2 macrophages, therefore participating in their balance in COVID-19 affected tissues such as airway and lung epithelia [95]. In an experimental model of rheumatoid arthritis, O_3 reduced the level of TNF-α and IL-12 from synovial immune cells and increased the anti-inflammatory cytokine IL-10 [152]. The anti-inflammatory property of O_3 is more often exerted by its oxidized phospholipids, such as 4-HNE, which is a powerful activator of Nrf2 and HO-1 synthesis, as reported in BV-2 microglial cells [153].

The effect of O_2-O_3 therapy, as in O_2-O_3-AHT, greatly affects the homeostasis of $CD4^+CD25^+Foxp3^+$ T regulatory (Treg) cells, as reported in a multiple sclerosis model. A significant enhancement in Treg cells, in microRNAs miR-17, miR-27, in IL-10 and TGF-β, was recently observed [31], so rescuing the normal Treg cells presence, as their number is reduced during COVID-19 [154]. The use of O_2-O_3-AHT is safe, as reported by the negligible effect on neutrophil function [155]. Moreover, past reports showed the immuno-regulatory action of O_3 on mast cells *in vitro* [156]. Due the fundamental involvement of mast cells in COVID-19 pathogenesis [157], this issue should be particularly worth of further investigation.

The role of O_3 in dampening or modulating the severe inflammatory response occurring in COVID-19, is particularly complex and involves a plethora of O_3-generated mediators in the blood, which directly or indirectly exert their action on innate and acquired immunity. Yet, O_3 in itself exerts a leading role in immunity, at least *in vitro*. Noticeably, the recent study from Umut Kan Kucucsezer and colleagues, reported that peripheral blood mononucleate cells (PBMs), withdrawn from healthy donors, when treated with medical O_3 in doses from 1.0 μg/ml to 50 μg/ml, did not show cytotoxicity at the lowest doses (<10 μg/ml), assessing that direct exposure with gaseous O_3 at high concentration may be toxic but, on the contrary, low doses of O_3 stimulated the development of the $CD3^-$, $CD16^+$-$CD56^+$ NK cells and the expression of the marker CD107a in those cells, so assessing an immuno-modulatory and anti-inflammatory action triggered by low doses of O_3, probably a clue of the hormetic principle [158-160].

The evidence that O_3 modulates immunity was reported also by its anti-microbial activity, for example against *Klebsiella pneumoniae* by enhancing innate immunity and MIP-2 production and for its anti-parasite activity *in vivo* [161]. Cabral and coworkers demonstrated that 20 μg/ml (topical) or 30 μg/ml (intraperitoneal) O_3 in BALB/c mice previously infected with $1x10^5$ promastigotes of *Leishmania amazonensis* (MHOM/BR/1977/LTB0016), reduced the parasite number, increased the leukocyte number and M1 macrophages arginase and noticeably triggered the wound repair mechanisms and collagen synthesis [162]. Furthermore, recent evidence has reported that the farnesoid receptor (FXR) regulates macrophage switching to a anti-inflammatory phenotype following O_3 exposure, as observed in ($FXR^{-/-}$) mice, where a prolonged oxidative stress leads to NF-κB-caused NO, increase and enhanced levels of TNF-α, IL-1β, CCR2, CL2, CC3CR1, and CC3L1 [163].

The same Nrf2/Keap-1/ARE signaling has a leading role in the modulation of cytokine storm, as outlined by some authors suggesting Nrf2 as an issue of pharmacological targeting [164]. In this perspective, it is conceivable to suggest the hypothesis that O_3 may exert a major anti-inflammatory effect via the activation of the Nrf2/Keap-1/ARE signaling. Actually, Nrf2 is able to suppress pro-inflammatory cytokine expression in macrophages, particularly IL-6 and IL-1β, as reported also in recent clinical studies where medical O_3, used to treat elderly people hospitalized in ITUs with COVID-19, significantly decreased IL-6 levels in the bloodstream [3,165]. Inhibition of inflammation by O_3 regards therefore medical hematological O_3 in the clinical course of an illness. As O_3 is a highly reactive substance, its anti-inflammatory potential can be retrieved only with proper and sound protocols, as empirical attempts usually fail in giving encouraging outcomes [166-168].

On the other hand, the immune pathogenesis of COVID-19 appears particularly complex. In this sense, the immunological context in which O_3 operates to reduce the immune impact of COVID-19 has to be further assessed, for example by highlighting the different pathogenesis of COVID-19 in various individuals. Post-mortem lung tissue biopsies in COVID-19 patients have outlined different kinds of manifestations in the immune disorder and dysregulation known as “cytokine storm” and any manifestation shared an increase in systemic inflammatory cytokines, such as IL-6 and a pro-thrombotic state, expressing high levels of ICAM-1, and sometimes showing, in the lung tissue, ficolin 3 (FCN3) in hyaline membrane, IL-1 and TNF-α and abundant mast cells /CD117 +) or alternatively a marked Th2-response, represented by high CD8 + cells, IL-4, IL-13 and tissue-related TGF-α [169]. At least two different kind of immune response may occur, a Th-1 or a Th-2 mediated immunity. In this context, O_3 may exert a complex immuno-modulatory activity, usually via O_3-generated bioactive metabolites, such as 4-HNE, which may have an anti-inflammatory role.

At least in obese patients and in adipocytes, 4-HNE was reported to regulate the genetic expression of TNF-α via the activation of the transcription factor ETS1 and the microRNA miRNA29b [170].

The role of 4-HNE in reducing inflammation, by inhibiting NF-κB, is well known [171], and furthermore 4-HNE can inhibit the production of TNF-α and IL-1β from monocytes activated by bacterial LPS, via the inhibition of the p38MAPK and ERK1/ERK2 signaling [119]. It is possible to speculate, therefore, that the anti-inflammatory activity of O_3 in COVID-19, may be fundamentally exerted by aldehydes derived from O_3-oxidized lipids in cells and the blood. Moreover, it has to be taken into account that innate immune cells may produce endogenous O_3, though in particular conditions [36,172,173]. Endogenous O_3 and O_3-caused lipid mediators are powerful anti-inflammatory tools. Cyclopentenone isoprostanes, which may be produced by O_3 interactions with lipids, are strong inhibitors of the inflammatory response in macrophages [174]. Some oxysterols, such as 25-hydroxycholesterol, have anti-inflammatory properties, being able to dampen the IL-1 mediated inflammation, downstream of TNF-α activation [175].

4. The role of ozone in coagulation and thrombotic mechanisms

So far, we have outlined that the main way by which O_3 would act against SARS-CoV2 infection and more exactly towards COVID-19 reducing its clinical impact, involves the activation of the anti-oxidant endowment of infected cells, i.e. scavenging enzymes and transcription factors, probably via a mild stress, which in turn activates ROS signaling and triggers the expression of a survival and anti-inflammatory response [176]. This may result a good hypothesis, as several reports have shown the ability of little or moderate dosages of gaseous O_3 into the blood to promote an anti-oxidant response in the organism, fundamentally targeting the Nrf2/keap-1/ARE pathway and HO-1 expression. Furthermore, O_3 may target also the complex nitric oxide/inducible nitric oxide synthase (NO/iNOS) pathway [177]. The highly widespread belief that COVID-19 may be fundamentally an endothelial-prothrombotic disease, as reported by the observation that NO, statins and ACE inhibitors are able to induce a less severe manifestation of COVID-19, reducing exacerbation, has currently set on the spotlight the role of NO in treating COVID-19 [178].

It is well known that NO is released by endothelia to prevent platelet-mediated thrombosis and to hamper new platelets recruitment in the thrombus formation [179]. Recent reports showed that, standing GSH depletion and hypoxic stimuli, NO simulates HO-1 production [180], an evidence strengthening the result that HO-1, the major anti-thrombotic mediator, is released by the Nfr2-mediated anti-oxidant activity and by NO, which in turn cross talk with HIF-1α signaling. Actually, while mild NO levels (usually $\leq$ 400 nmoles/L) are reported to promote HIF-1α proteasome-mediated degradation, impairing HIF-1α signaling, high NO doses ($\geq$1.0 mol/L) stabilize HIF-1α, even during normoxic conditions [181]. It is tempting to speculate that during COVID-19 a finely regulated interplay NO-HO-1-HIF-1α, more that a gigantic income of O_2, is the leading mechanism preventing vascular disorders and thrombosis reported in severe COVID-19.

Table 2 summarizes some of the major evidence on the effect of O_3 on ischemia/reperfusion injury models in laboratory animals and *in vitro* studies [181-190]. Besides to exacerbation in the pro-oxidant and pro-inflammatory status, COVID-19 is characterized by severe disorders in the endothelia-coagulation and pro-thrombotic system [191-195]. The ischemia/reperfusion (I/R) injury model represents a reliable experimental bench to investigate the activity of O_3 and its mediators on vascular-endothelial and thrombotic processes [196].

Pioneering studies conducted by Rokitansky et al., in 1981, evaluated the role of O_3 in modulating the production of 2,3-diphosphoglycerate (2,3-DPG), a fundamental factor for platelet thrombogenic function [78,197,198]. Factors enhancing blood oxygenation affect many mechanisms involving the physiology of vascular endothelia. A synergistic action exists between the Nrf2 activity and the role of HO-1, particularly in the cardiovascular function, therefore the anti-thrombotic and vascular-protective activity of HO-1 is potentiated by a positive synergistic loop from the O_3-mediated action on Nrf2 [199]. Several research studies, in laboratory animals, showed that the administration of O_3 in I/R models, often reduced the impact of the I/R-mediated damage and triggering an anti-oxidant response. However, the condition and O_3 dosages used in I/R models may be largely limitant, as O_3 exerts its action in a very complex system. Elsurer et al., submitted male Wistar rats to axillary artery ligation, causing ischemia for 3 hrs and then reperfusion for 24 hrs. O_3 after ischemic injury was administered intra-peritoneally (1.0 mg/kg, as an O_2/O_3 gas mixture 97% O_2 and 3% O_3, 3 L min^{-1}, with O_3 = 60 μg/ml). Rats treated with O_3 only (without I/R) reported only 8% tissue damage, respect to the 17% of sole I/R and 15% of ischemia + O_3 [185]. Rats treated with O_3 also in the I/R model, showed a marked increase in MDA, protein carbonyl (PCO), total antioxidant capacity (TAC), SOD, GSH-Px and catalase [185]. Onal and colleagues induced an I/R injury in male Wistar rats occluding the superior mesenteric artery for 60 min (ischemia), followed by 2 h of reperfusion. In the group pre-treated with O_3, using 3 L/min of an O_2 (97%)–O_3 (3%) gas mixture with 60 μg/mL O_3, volume 3.2–4.2 ml, the animals exhibited a decreased intestine mucosa injury and increased total antioxidant capacity (TAC), SOD, glutathione peroxidase and catalase [200].

The role of NO in I/R injury models is supported by the observation that administering NO-donors or compounds able to enhance NO

Table 2
Major historical studies on the effect of O_3 on ischemia/reperfusion (I/R) injury models.

Research model function	Rationale and method	Ozone method	Main results	References
Rat	Male Wistar rats (8–10 weeks old, 250–280 g weight) Renal I/R	2.5–2.6 ml O_2/O_3 mixture ozone concentration 50 mg/L, 0.5 mg/kg/rat) by rectal insufflation via a polyethylene cannula	↓ Damage score ↑ Serum creatinine, blood urea nitrogen (BUN) SOD, MDA, MPO	[182]
	Male Wistar rats Liver I/R 90 min right-lobe hepatic ischemia and 90 min reperfusion.	O_3 as 1.0 mg/kg. 10 O_3 treatments, 1/day, 5.0–5.5 ml O_3 50 μg/ml	↓ I/R caused injury, H_2O_2 ↑ SOD, GSH	[183]
	Sprague-Dawley rats I/R model unilateral nephectomy with 45 min ischemia and 24 hrs reperfusion	O_3 post-conditioning 2 mg/kg	↓ I/R induced damage, IL-1β, IL18, caspase 1, 11 ↑ Serum creatinine, BUN	[184]
	Wistar rats I/R axillary artery ligation 3 hrs, reperfusion 24 hrs	O_2-O_3 mixture (97% O_2, 3% O_3, O_3 60 μg/ml)	↑ CAT, SOD, GSH-Px, MDA, PCO, TAC ↓ I/R caused damage, TOS	[185]
	Male Wistar rats renal ischemia 30 min, reperfuson 3 hrs	15 O_3 treatments for rectal insufflation (50 μg/ml) O_3	↑ Renal function (plasma clearance of *p*-ammino hippurate), SOD ↓ Phospholipase A, necrotic damage	[186]
	Male Wistar rats liver right lobe acute ischemia 90 min, reperfusion 24 hrs	10 O_3 treatments, one per day, 5.0–5.5 ml concentration 50 μg/m1	↓ Transaminase level, xanthine accumulation, increase in ADA from ischemia	[187]
	Male Wistar rats liver right lobe acute ischemia 90 min, reperfusion 90 min	15 O_3 treatments, one per day, 5.0–5.5 ml concentration 50 μg/m1	↓ Ischemia induced amage	[188]
	Male Wistar rats liver right lobe acute ischemia 90 min, reperfusion 90 min	15 O_3 treatments, one per day, 5.0–5.5 ml concentration 50 μg/m1	↑ Liver protection via mechanisms producing NO	[189]
SH-SY5Y cell line	*In vitro* neuroblastoma cell line SH-SY5Y in erebral I/R model	4×10^5 SH-SY5Y cells in 1 ml medium with 1 ml O_3 in 10 ml	↓ Mitochondria-mediated apoptosis ↑ SOD, CAT, GSH-Px	[190]

ADA; adenosine deaminase; CAT: catalase; GSH-Px: glutathione peroxydase; MDA: malonyldialdheyde; MPO: myeloperoxidase; PCO: protein carbonyl; SOD: superoxide dismutase; TAC: total antioxidant capacity; TOS: total oxidant status.

production before inducing ischemia, the injury following I/R is greatly reduced [201]. The O_2/O_3 mixture used to inject O_3 in the blood is able to activate the endothelial nitric oxide synthase (eNOS) [202,203], therefore the release of NO is directly triggered by O_3, or by its end products [204]. Furthermore, NO up-regulates the expression of HO-1, so enhancing the cardiovascular protective action of HO-1 [205]. An increase in NO following O_3 treatment was reported very recently also by Yasemin Dere Günal and colleagues in an ischemia/reperfusion (I/R) injury model in male Wistar rats [206]. However, in these I/R injury models with rats, protocols are crucial to envisage a positive result from using O_3 as a profilactic agent against I/R damage. In the Turkish experience, Dere Günal and coworkers reported on male Wistar rats exacerbation in their I/R model , with reduction in SOD, caused by O_3 pre-conditioning, yet the authors did not detail their I/R model and used 0.7 mg/kg of an O_2/O_3 (95% /5%, i.e. 0.35 μg/ml O_3) for 20 min, whereas Ozkan Onal et al., from the Department of Anesthesiology and Reanimation, Selçuk University Medical Faculty, Konya, Turkey, used on male Wistar rats, an O_3 pre-conditioning represented by 3.2–4.2 ml for each animal of a gaseous mixture O_2/O_3 (97% /3%) having 60 μg/ml O_3, much more close to dosage ranges (45–50 μg/ml) used in humans for COVID-19 (see also Tables 1A and 1B) [200]. The correct O_3 protocol is mandatory to earn positive outcome in the use of this gas. O_2-O_3-AHT may lead to the formation of NO and oxidants causing the production of 3-nitrotyrosine as a fundamental signal to activate a mild oxidative stress and inhibit thrombotic events, as NO is in itself an anti-thrombotic signal [179,207]. NO has a role in aspirin-induced thrombolysis [208] and moreover NO regulates tissue factor (TF) for coagulation, which is activated during COVID-19 exacerbation leading to disseminated intravascular coagulation (DIC) and other vascular disorders (DIC) [209,210]. We do not know if the rapid decrease in plasma D-dimer observed in patients with COVID-19 treated with O_2-O_3-AHT [1-3], should be an effect of NO signaling caused by O_3 on endothelia, yet O_3 on HUVECs allows the production of NO [211,212].

Furthermore, in ischemia/reperfusion (I/R) injury models, O_3 proved to reduce and prevent the IR-caused damage, usually by activating the oxidative stress response. Endothelial dysfunction plays a major role in I/R injury [212]. Wistar rats, undergoing superior mesenteric artery occlusion for 1 h and reperfusion for two hours, when administered with 1.0 mg/kg of O_3 increased intestinal tissue levels of SOD, glutathione peroxidase and catalase [200]. Moreover, tissue protection from I/R injury by O_3 may involve the activation of iNOS. Foglieni et al., recovered the kidney functional activity in rats undergoing unilateral nephrectomy by using 1 ml autologous blood with 5 ml of an O_2/O_3 mixture (50 μg/ml O_3) and observed that the production of iNOS by β-NADPH diaphorase in glomerular capillaries increased significantly, following ischemic injury [213]. Although many data about ozone in I/R injury refer to the gaseous airborne O_3 as a pollutant, inhaled O_3 reduces the plasminogen activator inhibitor-1 (PAI-1), therefore enhancing the presence of tissue plasminogen (tPA), then enabling the reduction of clots and thrombi and promoting fibrinolysis [214], i.e. disrupting intravascular thrombi, particularly in microcirculation [215]. Interestingly, PAI-1 increases also when SOD and glutathione peroxydase (GSH-Px) are enhanced, as occurring following treatment with O_3 [216].

It is tempting to speculate that RES produced by O_3, such as 4-HNE, may play a crucial role in the complex balance pro-thrombotic/anti-thrombotic signals following O_3 exposure, as its dosage has a particular stringency in this context [217]. The anti-oxidant property of O_3 is therefore fundamental to prevent ROS formation in venous thrombosis [218]. Streptozotocin-induced diabetic male Sprague-Dawley rats, receiving 10 treatments with O_2/O_3 50 μg/ml O_3, increased the levels of GSH, GSH-Px, SOD and catalase, besides to reducing total peroxides and the endothelial damage [219]. Di Filippo and colleagues treated Sprague Dawley rats, undergoing an acute myocardial ischemia/reperfusion injury with 100, 150 and 300 μg/Kg of an O_2/O_3 mixture injected intraperitoneally [220]. Rat heart underwent exteriorization, then a fine silk ligature was used to occlude the left anterior coronary artery (a device called LADCA), in proximity to its origin. In this condition, rats were maintained under mechanical ventilation with ambient air (rate 54 S/min, stroke volume ranging from 1.0 to 1.5 ml/0.1 Kg) and a positive ending respiratory pressure estimated from 0.5 to 1.0 H_2O cm. Rats underwent 25 min ischemia and 2 h of reperfusion [220]. The I/R injury with LADCA exhibited significant difference ($p < 0.001$) in the level of nitrotyrosine, and IL-6, CXCL8 in infarcted rats if treated with 300 μg/kg of the mixture O_2/O_3 respect to controls and also the expression of immune cell expressing CD68, CD8 and CD4 was completely different in O_3 treated animals [220]. Furthermore, the expression of caspase 3 in myocardial tissue decreased at 150 μg/ml O_3 and much more at 300 μg/ml O_3 [220]. In addition, using the same I/R injury models on rats, this research team observed that in animals treated with the O_2/O_3 mixture, an increase in the expression of CD34 + and CD117/c-kit in myorcardial tissue and of eNOS, rapidly occurred, whereas the pre-treatment with a known eNOS inhibitor (30 mg/kg N5-(1-Iminoethyl)-L-ornithine dihydrochloride (L-NIO) subcutaneous injection), suppressed the protective role of O_3 in inducing eNOS [221]. The numerous I/R injury models suggest altogether the fundamental role of O_3 as a small molecule able to target fundamental genes involved in I/R injury, such as LCN2, CCL2, HP, HMOX1, CCL7, CCL4, and S100A8 and several micro-RNAs, as O_3 dampens the pro-inflammatory machinery in the I/R injury model by inhibiting the NLRP3-mediated inflammation and enhancing the Nrf2/Keap1/ARE pathway [222,223].

The activity of O_3 in reducing inflammatory may be independent from the methodology used in injecting medical O_3, as very recently Fernandez-Cuadros and coworkers, observed reduced CRP, IL-6 and D-dimer in COVID-19 patients treated with rectal ozone therapy [10].

In conclusion, the way by which O_3 may counteract COVID-19 associated thrombosis and disseminated intravascular coagulation (DIC) accounts on a) reducing the ROS impact on pro-thrombotic signals; b) activating the NO/iNOS/eNOS pathway, via the HO-1, and usually by O_3 formed mediators in the hormetic ranges. Yet, more insightful evidence is needed to assess the anti-thrombotic role of O_3 in COVID-19. The elucidation of the mechanisms of action of O_3 in the complex endothelia-plasma cross-talk may provide important clues about its pharmacological action.

5. Conclusions

Which is the potential of O_3 in treating COVID-19? Ozone is not a pharmaceutical drug but a small regulatory molecule able to generate bioactive mediators acting on the complex cross talk oxidative stress-inflammation-vascular function. The pharmacological activity of medical O_3 depends fundamentally on the ability of O_3-derived products to trigger a mild ROS signaling, or a mild mitochondria stress (mitohormesis) in order to activate an anti-oxidant response, driving the modulation of immunity towards anti-inflammatory mechanisms and leading to a wide inhibition of pro-thrombotic events. Despite all O_3-induced byproducts are pro-oxidant molecules, able to induce oxidative damage, their moderate expression leads towards a survival response, particularly in sick or ill subjects, rendering O_3 a powerful tool against COVID-19. The numerous clinical reports, showing the ability of O_2-O_3 AHT to greatly reduce the exacerbation of COVID-19 pneumonia or ARDS, are promising news to address successfully SARS-CoV2 pandemic.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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