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Ozone: a natural bioactive molecule with antioxidant property as potential new strategy in aging and in neurodegenerative disorders.

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Highlights

- Aging and neurodegenerative diseases are characterized by complex and multifactorial aetiology and challenges regarding treatments efficacy and costs persist.
- Oxygen-ozone (O_2-O_3) therapy is a non-invasive, non-pharmacological, low cost and no-side effect procedure based on antioxidant capacity of O_3.

- A plethora of scientific evidence supports other crucial properties of O_3 in physiopathological processes.
- Nrf2 is one of the principal molecular signalling on which O_3 exercises its antioxidant/anti-apoptotic/pro-autophagy effects, involved in aging and in neurodegenerative disorders.
- This review provides a consistent rationale to implement future clinical studies to apply the O_2-O_3 treatment along with other antioxidants (polyphenols, mushrooms) in aging and in neurodegeneration.

Abstract

Systems medicine is founded on a mechanism-based approach and identifies in this way specific therapeutic targets. This approach has been applied for the transcription factor nuclear factor (erythroid-derived 2)–like 2 (Nrf2). Nrf2 plays a central role in different pathologies including neurodegenerative disorders (NDs), which are characterized by common pathogenetic features. We here present wide scientific background indicating how a natural bioactive molecule with antioxidant/anti-apoptotic and pro-autophagy properties such as the ozone (O_3) can represent a potential new strategy to delay neurodegeneration. Our hypothesis is based on different evidence demonstrating the interaction between O_3 and Nrf2 system. Through a meta-analytic approach, we found a significant modulation of O_3 on endogenous antioxidant-Nrf2 ($p<0.00001$, Odd Ratio (OR)=1.71 95%CI:1.17-2.25) and vitagene-Nrf2 systems ($p<0.00001$, OR=1.80 95%CI:1.05-2.55). O_3 activates also immune, anti-inflammatory signalling, proteasome, releases growth factors, improves blood circulation, and has antimicrobial activity, with potential effects on gut microbiota. Thus, we provides a consistent rationale to implement future clinical studies to apply the oxygen-ozone (O_2-O_3) therapy in an early phase of aging decline, when it is still possible to intervene before to potentially develop a more severe neurodegenerative pathology. We suggest that O_3 along with

other antioxidants (polyphenols, mushrooms) implicated in the same Nrf2-mechanisms, can showed neurogenic potential, providing evidence as new preventive strategies in aging and in NDs.

Keywords: Neurodegenerative Disorders; nuclear factor (erythroid-derived 2)–like 2 (Nrf2); Ozone (O_3); Oxygen-Ozone (O_2-O_3) therapy; antioxidant system; stress oxidative biomarkers.

1. Introduction

Life span has almost doubled in the last century (WHO, 2011, Wyss-Coray, 2016), and consequently aging-specific diseases are becoming prevalent (Moskalev et al., 2017). However, the pathophysiologic mechanisms underlying most of them are still poorly understood and challenges regarding treatments efficacy and costs persist.

Neurodegenerative diseases (NDs, Alzheimer's disease, AD; Parkinson disease, PD; amyotrophic lateral sclerosis, ALS, Huntington Disease, HD) are the most prevalent cognitive and motor disorders of the elderly. These aging-specific diseases are characterized by the loss of homeostasis during aging, leading to low-grade stress by pathologic formation of Reactive Oxygen Species (ROS), chronic inflammation, mitochondrial dysfunction and metabolic unbalance (Dugger, Dickson, 2017). In addition, these pathophenotypes are determined by abnormal aggregation of specific proteins (Yanar et al., 2020), given the connection between excessive ROS accumulation and impairment in proteostasis network.

Despite their distinct causative factors and clinical symptoms, these diseases as well as aging have common pathogenetic features (Aso et al., 2012). This implicates potentiality in the identification of therapeutic targets on a set of disease phenotypes and physiological conditions that are mechanistically linked. Thus, contrary to a hitherto linear approach that considered one disease, one medicine, to date there is a need for a new concept of therapy condensed as "several diseases, one medicine". In this way, diseases are diagnosed not only by clinical symptoms, but mainly by the underlying molecular signatures (Goh et al., 2007). Based on this network medicine approach, (Cuadrado et al., 2018, Cuadrado et al., 2019) reported extensive evidence about the central role playing by nuclear factor (erythroid-derived 2)–like 2 (Nrf2). Nrf2 is widely known and

investigated as a master regulator of multiple cytoprotective responses and as a key molecular node within a cluster of a wide spectrum of diseases, including NDs. Moreover, Nrf2 activation is impaired in aging by the involvement of microRNA (Zhang, H. et al., 2015, Schmidlin et al., 2019, Silva-Palacios et al., 2018). This suggests that Nrf2 could represent a common therapeutic and systems medicine target, for aging and for its related disorders. Nrf2 can transcriptionally modulate the cytoprotective genes belonging to the vitagene network. This network regulates endogenous cellular defense mechanisms, and involves redox sensitive genes such as members of the Heat Shock Proteins (HSP) family (*heme-oxigenase HO-1*, *Hsp70*), but also sirtuins and the thioredoxin (Trx)/thioredoxin reductase (TrxR1) system (Calabrese, V. et al., 2010).

Based on this rationale, in this review we present wide scientific background indicating how a natural bioactive molecule with antioxidant property such as the ozone (O_3) can be indicated as a potential new strategy to delay neurodegeneration. This hypothesis is based on the widely demonstrated evidence regarding the interaction between O_3 and Nrf2 (Galie et al., 2018, Siniscalco et al., 2018, Re et al., 2014, Vaillant et al., 2013). We first describe the relevant, well known and documented molecular mechanisms related to antioxidant/anti-apoptotic/pro-autophagy processes targeted by the O_3 administration *via* Nrf2 biological pathway. Secondarily, we report a list of the main stress oxidative biomarkers modulated by the O_3 treatment *via* Nrf2 and that, in turn are strongly involved in NDs pathophysiology as well as in aging mechanisms. Different meta-analyses have been performed to demonstrate the effect in terms of Odd Ratio (OR) of O_3 on endogenous antioxidant-Nrf2 and vitagene-Nrf2 systems.

We thus provide scientific evidence to build a consistent rationale for apply for the first time the Oxygen-Ozone (O_2-O_3) therapy in an early phase of aging decline, when it is still possible to intervene, before to develop a potential neurodegenerative pathology.

2. The Ozone (O_3) molecule and the Oxygen-Ozone (O_2-O_3) therapy

O_3 is a triatomic gaseous molecule which has been using as a powerful oxidant in medicine for more than 150 years (Elvis, Ekta, 2011). In nature, O_3 is generated during storms due to the

electrical discharges of the rays that react with atmospheric O_2 to produce O_3. In humans, a revolutionary discovery leaded to demonstrate that neutrophils isolated from human peripheral blood and coated with antibodies can catalyse the generation of O_3 by a water oxidation pathway, leading to efficient killing of bacteria (Wentworth et al., 2002, Babior et al., 2003, Lerner, Eschenmoser, 2003).

In 1785, Van Mauren was the first identifying the distinctive odor of O_3. The actual gas was later discovered by the German chemist, Christian Friedrich Schonbein at the University of Basel in Switzerland on March 13th, 1839 when working with a voltaic pile in the presence of O_2 (Altman, 2007). Friederich noticed the emergence of a gas with an electric and pungent smell, and named it ozone, which is derived from the Greek word for smell (Bocci, V., 2011). ~~In 1860, Jacques-Louis Soret, a Swiss chemist demonstrated that the O_3 was made up of three atoms of oxygen (Altman, 2007).~~ O_3 was used as first antiseptic for operating rooms and to disinfect surgical instruments in 1856, and in 1860 the first O_3 water treatment plant was built in Monaco to disinfect water (Altman, 2007). Nikola Tesla patented the first portable O_3 generator in 1896 in the United States. The physicist, Joachim Hansler invented the first reliable O_3 generator, and this was the breakthrough in the use of O_3 for medical applications. This invention is considered the prelude to the ozonated autohemotherapy procedure and served as the basis for O_3 therapy expansion over the last 40 years.

~~The use of O_3 in the clinical practice was introduced in the past century (Wolff, 1915). During the World War I, from 1914 to 1918, doctors used O3 to successfully treat post-traumatic gangrene in German soldiers, bone fractures, inflammations, and abscesses (Bocci, 2011). Due to its prophylactic proprieties, O3 was also used to prevent infections in local medical procedures and to control wound infections (Merin et al., 2007).~~

The O_2-O_3 therapy is a non-invasive, non-pharmacological, no-side effect and low-cost procedure applied in medicine for the treatment of more than 50 pathological processes, whose alterations in endogenous oxidative-antioxidative balance play a crucial role. ~~Importantly, d~~Different clinical trials evidenced the effectiveness of this therapy in the treatment of degenerative

disorders such as multiple sclerosis (Smith et al., 2017, Delgado-Roche et al., 2017, Ameli et al., 2019), but also cardiovascular, peripheral vascular, neurological, orthopaedic, gastrointestinal and genitourinary pathologies (Bocci, V., 2011, Elvis, Ekta, 2011, Re et al., 2008, Bocci, V., 2012, Smith et al., 2017, Braidy et al., 2018); fibromyalgia (Moreno-Fernandez et al., 2019, Tirelli et al., 2019); skin diseases/wound healing (Fitzpatrick et al., 2018, Wang, X., 2018); diabetes/ulcers (Martinez-Sanchez et al., 2005, Guclu et al., 2016, Rosul, Patskan, 2016, Izadi et al., 2019, Ramirez-Acuna et al., 2019); infectious diseases (Smith et al., 2017, Mandhare et al., 2012, Song et al., 2018), including the recent global pandemic disease of coronavirus disease 2019 (COVID-19) (Zheng et al., 2020); dentistry (Isler et al., 2018, Khatri et al., 2015, Srikanth et al., 2013, Azarpazhooh et al., 2009); lung diseases (Hernandez Rosales et al., 2005); osteomyelitis (Bilge et al., 2018). The potential role of O_2-O_3 as an adjuvant therapy for cancer treatment has been also suggested in *in vitro* and animal studies as well as in isolated clinical reports (Clavo et al., 2018).

At present, we have commenced a randomized double-blind clinical trial with the aim to test the efficacy of this therapy in a cognitive frailty cohort, a grant approved by the Italian Minister of Health (RF-2016-02363298). This pilot study will permit to validate the O_2-O_3 therapy in an early phase of cognitive decline, when it is still possible to intervene, before to develop a potential neurodegenerative pathology.

To date, the O_2-O_3 therapy acquires a further prestigious significance, after the medicine Nobel prize for "discovery of how cells sense oxygen" in 2019. Indeed, O_2 is the most vital element required for human life and it is the key to good health; O_3 is O_2 with an extra molecule added. The O_2 availability affects genes expression of different factors (HIFs, Hypoxia Inducible Factors), leading to the activation of trophic proteins (VEGF, Vascular Endothelial Growth Factor; PDGF, Platelet-derived growth factor) and consequently to specific biological processes, including erythropoiesis, angiogenesis and anaerobic glucose metabolism (Zhou et al., 2019). O_3 plays a role of cellular adapter to hypoxia, because it is well known its effects in increasing the levels of VEGF,

PDGF, HIF (Curro et al., 2018, Zhang, J. et al., 2014, Re et al., 2010), exactly as the cell does when there is no O_2 available.

3. Focus on the biological activities of the ozone (O3): antioxidant property

Oxidative stress is a condition where ROS and Nitrogen Species (RNS) production exceeds the cellular antioxidant defence system, leading to the imbalance between the two systems and this may contribute to the neuronal damage and the abnormal neurotransmission. It is widely known its implication in the pathogenesis and progression of NDs (Singh et al., 2019). Brain and mitochondria are the most involved systems due to their high sensitivity to oxidative damage caused by free radicals. Oxidative damage may impair the cells in their structure and function, being cause and effect of a mitochondrial reduced activity. The damage is not confined to the brain but also evident in peripheral cells and tissues.

ROS and RNS are also major factors in cellular senescence that leads to increase number of senescent cells in tissues on a large scale (Liguori et al., 2018). Cellular senescence is a physiological mechanism that stops cellular proliferation in response to damages that occur during replication. Senescent cells acquire an irreversible senescence-associated secretory phenotype (SASP), involving secretion of soluble factors (interleukins, chemokines, and growth factors), degradative enzymes like matrix metalloproteases (MMPs), and insoluble proteins/extracellular matrix (ECM) components.

Nrf2 is a member of the CNC-basic leucine zipper (CNC-bZIP) family of transcription factors. Under basal condition, Nrf2 binds to its repressor Keap1 (Kelch-like ECH-associated protein 1), an adapter between Nrf2 and Cullin 3 protein, which leads to ubiquitination followed by proteasome degradation. This Keap1-mediated degradation activity requires two reactive cysteine residues (Cys273 and Cys288).

When O_3 is administrated, it dissolves immediately in the plasma/serum and it reacts with PUFA (polyunsaturated fatty acids), leading to the formation of the two fundamental messengers: hydrogen peroxide (H_2O_2) as a ROS and 4-hydroxynonenal (4HNE) as a lipid oxidation product

(LOP) (Bocci, V. et al., 1998) (Figure 1). ROS are the early and short-acting messengers, while LOPs are late and long-lasting messengers. LOPs diffuse into all cells and inform them of a minimal oxidative stress. After the oxidative/electrophilic stress challenge (4HNE, (Ishii et al., 2004), other aldehydes (Levonen et al., 2004), induced by O_3 (Galie et al., 2018, Siniscalco et al., 2018, Re et al., 2014, Vaillant et al., 2013), modification of the cysteine residues of Keap1 (S-HNE or—S—S) inhibits ubiquitin conjugation to Nrf2 by the Keap1 complex (Brigelius-Flohe, Flohe, 2011), provoking the nuclear accumulation of Nrf2. Once in the nucleus, Nrf2 dimerizes and binds to cis-acting DNA AREs (Antioxidant Response Elements) in genes such as *Heme Oxygenase 1* (*HO-1*), a gene encoding enzyme that catalyses the degradation of heme in carbon monoxide (CO) and free iron, and biliverdin to bilirubin. CO acts as an inhibitor of another important pathway NF-κB (Nuclear Factor Kappa B Subunit 1) signalling, which leads to the decreased expression of pro-inflammatory cytokines, while bilirubin also acts as an important lipophilic antioxidant. Furthermore, HO-1 directly inhibits the pro-inflammatory cytokines and activating the anti-inflammatory cytokines, thus leads to balancing of the inflammatory process (Ahmed, S. M. et al., 2017). Our research group confirmed that mild ozonisation, tested on *in vitro* systems, induced modulation of genes, including *HO-1* (Scassellati et al., 2017). (Figure 1).

In addition, Nrf2 regulates also the constitutive and inducible expression of antioxidants including, but not limited to, Superoxide Dismutases (SOD), Glutathione Peroxidase (GSH-Px), Glutathione-S-Transferase (GST), Catalase (CAT), NADPH quinone oxidoreductase 1 (NQO1), phase II enzymes of drug metabolism and HSPs (Galie et al., 2018, Bocci, V., Valacchi, 2015, Pedruzzi et al., 2012) (Figure 1).

A further mechanism involves casein kinase 2 (CK2), another regulator of the Nrf2 activity through its phosphorylation. It has been demonstrated that O_3 influenced the CK2 levels together with Nrf2 phosphorylation, reducing oxidative stress and pro-inflammatory cytokines in multiple sclerosis patients (Delgado-Roche et al., 2017). Similarly, O_3 inhibits oxidative stress through

inhibition of the mitogen-activated protein kinase phosphatase (MAPK) 1 signalling pathway (Wang, L. et al., 2018) (Figure 1A).

Oxidative stress is one of the major drivers of protein misfolding that, accumulating and aggregating as insoluble inclusions can determine neurodegeneration (Hohn et al., 2020, Knowles et al., 2014). It is known that Nfr2 promotes the clearance of oxidized or otherwise damaged proteins through the autophagy mechanism (Tang et al., 2019). Interestingly, also O_3 can modulate the degradation protein systems, not only *via* Nrf2 pathway, but also *via* activation of the AMP-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) signaling pathway, as demonstrated in (Zhao, X. et al., 2018) (Figure 1B).

O_3 can protect against overproduction of nitric oxide (NO), when NO is a toxic oxidant. NO can rapidly react with other free radicals such as $O2^{-\bullet}$ to generate highly reactive oxidant peroxinitrite ($ONOO^-$) and other RNS, which in turn damages the biomolecules (e.g., lipids, protein, DNA/RNA), playing thus a key role in chronic inflammation and neurodegeneration (Massaad, 2011, Toda et al., 2009). It has been demonstrated that O_3 downregulates inducible nitric oxide synthase (iNOS), which generates NO (Manoto et al., 2018, Smith et al., 2017) *via* NF-κB signalling (Figure 1C).

4. Focus on the biological activities of the ozone (O3): anti-apoptotic mitochondrial property

Mitochondrial dysfunction is one of the main features of the aging process, particularly in organs requiring a high-energy source such as the heart, muscles, brain, or liver. Neurons rely almost exclusively on the mitochondria, which produce the energy required for most of the cellular processes, including synaptic plasticity and neurotransmitter synthesis. Mitochondrial disfunctions cause increase in ROS for lowered oxidative capacity and antioxidant defence, with consequent increased oxidative damage to protein and lipids, decreased ATP production and accumulation of DNA damage (Garcia-Escudero et al., 2013, Reutzel et al., 2020). Moreover, mitochondrial

bioenergetic dysfunction and release of pro-apoptotic mitochondrial proteins into the cytosol initiate a variety of cell death pathways.

Nrf2 transcribes several genes not only those implicated in antioxidant expression and energy regulation, but also those involved in mitochondria biogenesis: increases the mitophagy, mitochondrial levels of antioxidant enzymes, and resistance to redox regulated mitochondrial permeability transition pore opening (Holmstrom et al., 2016). Multiple lines of evidence have shown that Nfr2 activation is part of the retrograde response aimed at restoring mitochondrial functions after stress insults, and that the impairment of Nrf2 functions is a hallmark of many mitochondrial-related disorders (Shan et al., 2013).

It has been demonstrated that O_3 administration can act on specific mechanisms to promote cell survival and proliferation, blocking the apoptotic processes. In particular, O_3 decreases the expression of *caspases 1-3-9, HIFα, Tumor Necrosis Factor-a (TNF-α), Bcl-2-associated X protein (Bax), poly (ADP-ribose) polymerase 1 (PARP-1)* and *p53* genes (Figure 2) (Yong et al., 2017, Guclu et al., 2016, Wang, L. et al., 2018). Bax is located in the mitochondrial membranes and exerts pro-apoptosis effect through the mitochondrial pathway, promoting cytochrome C activation (Mac Nair et al., 2016); p53 and Caspase-3 are executive molecules of apoptosis by blocking cell cycle (Wang, J. et al., 2016). Enzymes such as SOD, CAT, and GSH-Px, can regulate p53, Bax and Bcl-2 (BCL2 Apoptosis Regulator) (Figure 2).

Moreover, O_3 stimulates the Kreb's cycle in the mitochondria by enhancing the oxidative carboxylation of pyruvate and stimulating the production of adenosine triphosphate (ATP) (Guven et al., 2008). It also causes a significant reduction of nicotinamide adenine dinucleotide (NADH), an increase of the coenzyme A levels to fuel the Kreb's cycle and oxidizes cytochrome C (Brigelius-Flohe, Flohe, 2011, Elvis, Ekta, 2011).

O_3 treatment was proven to reduce mitochondrial damage in a rat heart following ischemia-reperfusion (Meng et al., 2017), as well as in a rat brain and cochlea following noise-induced

hearing loss (Nasezadeh et al., 2017). Moreover, *in vitro*, O_3 increased the length of the mitochondrial cristae and the content of mitochondrial Hsp70 (Costanzo et al., 2018).

5. Pro-oxidation and antioxidant defence biomarkers influenced by ozone (O_3) and implicated in aging processes and in neurodegenerative disorders (NDs)

5.1 Stress-oxidant biomarkers modulated by the O_3 effect

A list of biomarkers (29 in total) implicated in oxidative stress, in endogenous antioxidant and vitagene systems are showed in Table 1. These biomarkers have been studied and found modulated after the O_2-O_3 therapy in more of 150 studies performed in different *in vivo* (human and animal models) and *in vitro* samples and conditions. In Table 1, we also reported the relative functions of these biomarkers.

From these 29 biomarkers, we focused, in this section, on those implicated in endogenous antioxidant-Nrf2 pathway (GSH; GSH-Px; glutathione reductase, GR; SOD; CAT; 4HNE; Advanced Oxidation Protein Products, AOPP in bold in Table 1). Where it was possible (available studies), we performed meta-analyses for these biomarkers on human (see supplementary material). The results showed a significant increased levels of the SOD-CAT-GSH-Px-GSH-GST-GR after O_3 administration (Figure 3, Random model, Z=6.15, p<0.00001, OR=1.71 95%CI:1.17-2.25; even after Bonferroni correction 0.05/6=0.0083). Similar results were obtained even considering single markers, except for GR (Z=1.04; p=0.30) and GSH (Z = 0.80, p=0.42). GR has been investigated only in two studies, coming from the same authors (Hernandez Rosales et al., 2005). Thus, there are not enough evidence on its single real involvement. Concerning GSH, Diaz-Luis et al., (Díaz-Luis et al., 2018) is the only study showing a negative effect of O_3. As we followed the criteria for which the data were extracted before and after O_3 treatment (see supplementary material), this study found an increased GSH levels after O_3 administration, only when the authors performed the comparisons with control group of healthy subjects (in a sort of postconditioning). Thus, if we eliminated this study, the results of the single meta-analysis of GSH highlighted its positive increase determined by the O_3 treatment (Z=2.30; p=0.02, data not shown).

High heterogeneity in effect size across the studies ($P < 0.00001$, $I^2 = 97\%$) was observed in these meta-analyses. This is essentially explained by the presence of different factors: the type of pathology, different concentration of O_3 linked to different administration procedures and duration time treatments, age of the sample (supplementary material Table 1S).

~~Although there are contrasting results, most of the studies agree on the decrease in tissue levels of free radicals (lipid peroxidation markers, Pro-oxidation biomarkers – for instance Thiobarbituric acid reactive substances, TBARS; Malondialdehyde, MDA; 4-HNE, Advanced oxidation protein products; AOPP), and increase in the tissue levels of antioxidants (antioxidant defense markers – for instance GSH; SOD; CAT; GSH-Px; TH; glutathione disulfide, GSSG; Ferric Reducing the Ability of Plasma (FRAP), after O_3 administration, maintaining in this way antioxidant- prooxidant balance by the O_2-O_3 therapy.~~

Interestingly, different studies have been performed on aging-specific conditions. A recent work (El-Mehi, Faried, 2020) demonstrated that antioxidant properties of O_3 can ameliorate age-associated structural alterations of the rat cerebral cortex, improving age- related oxidative stress reflected in the histopathological and immunohistochemical alterations. The authors detected severe structural and cellular neurodegenerative changes in the frontal cortex of the aged rats. O_3 administration produced significant downregulation of tissue Malondialdehyde (MDA), an index of oxidative stress, and upregulation of GSH, SOD and CAT. Similarly, O_3 influenced iNOS, caspase-3, glial fibrillary acidic protein (GFAP), Ki67 and acetylcholinesterase (AChE). These findings indicate reduction not only in oxidative stress, but also in apoptosis (down-regulation caspase-3) and in gliosisas (down-regulation GFAP), as well as improving in neurogenesis (upregulation of Ki-67 expression) and in cholinergic plasticity (decrease AChE activity). The authors suggest that O_3 might be useful for improving the age – related cognitive and memory deterioration, by increasing cholinergic communication.

Safwat et al. (Safwat et al., 2014) demonstrated that O_3 showed a beneficial effect on the aging reducing liver and kidney damage through its antioxidant property. O_3 was efficient in

elevating the reduced hepatic and renal GSH contents as well as in normalizing hepatic GSH-Px activity of aged rats. Moreover, O_3 succeeded in attenuating the elevated hepatic and renal MDA and protein carbonyls (PC) levels.

Another work (El-Sawalhi et al., 2013) reported that O_3 alleviated age-associated redox state imbalance, as evidenced by reduction of lipid and protein oxidation markers and lessening of lipofuscin deposition. Moreover, O_3 restored GSH levels in brain and heart tissues, and normalized GSH-Px activity in the heart tissue of the aged-rats. O_3 also mitigated age-associated energy failure in the heart and the hippocampus, improved cardiac cytosolic Ca(2+) homeostasis and restored the attenuated Na(+) , K(+) -ATPase activity in the hippocampus of these rats.

Similarly, prophylactic administration of O_3 in aged-rats normalized reduced GSH content, adenosine triphosphate/adenosine diphosphate ratio, mitochondrial SOD and complex IV (cytochrome-c oxidase) activities. O_3 improved glutathione redox index (GSHRI), complex I (NADH-ubiquinone oxidoreductase) and mitochondrial mtNOS activities, and attenuated the rise MDA and mitochondrial PC levels (Shehata et al., 2012).

5.2. Stress-oxidant biomarkers implicated in aging mechanisms

Several evidence support the involvement of these biomarkers influenced by the O_3 administration in the mechanisms of aging (Table 1). We prevalently focused on those implicated in the Nrf2 signalling (in bold in Table 1).

It has been reported that the levels of lipid peroxidation products, reactive carbonyl compounds, such as 4HNE, are increased in aging tissues (Csala et al., 2015), and this increase is positively correlated with age. Impaired protein function, manifested as an increase in PC, plays a crucial role in aging processes (Cabiscol et al., 2014). With increase of PC, the spontaneous carbonyl-amino crosslinking and accumulation were mostly irreparable changes associated with aging (Nowotny et al., 2014).

Several findings evidenced altered levels of AOPP in aging (Komosinska-Vassev et al., 2012, Rusanova et al., 2018, Qing et al., 2012, Silva et al., 2015, Muller et al., 2015). A recent work

investigated the antioxidant enzymes (GSH-Px, CAT, SOD), nonenzymatic antioxidants (GR), redox status (total antioxidant capacity, TAC, total oxidant status, TOS, oxidative stress index, OSI), and oxidative damage products (AOPP, MDA) in a healthy sample divided according to age: 2-14 (children and adolescents), 25-45 (adults), and 65-85 (elderly people). They demonstrated that salivary and blood antioxidant defense is most effective in adults. Contrarily, a progressive decrease in the efficiency of central antioxidant systems (↓GSH-Px, ↓SOD, ↓GSH, ↓TAC in erythrocytes and plasma vs. adults) was observed in the elderly. Both local and systemic antioxidant systems were less efficient in children and adolescents than in the group of middle-aged people, which indicates age-related immaturity of antioxidant mechanisms. Oxidative damage to proteins (↑AOPP) and lipids (↑MDA) was significantly higher in saliva and plasma of elderly people in comparison with adults and children/adolescents (Maciejczyk et al., 2019). Similarly, Cakatay et al. (Cakatay et al., 2008) found, in a young, middle-aged and elderly individuals, PCO and AOPP levels of the elderly and middle aged individuals higher compared with those of the young.

Although not involved in Nrf2 signaling but influenced by O_3 treatment, the increased oxidative damage to mitochondrial DNA (mitDNA) with the OH8dG (8-hydroxydeoxyguanosine) formation, represents the most common hallmark of the aging brain, marker of oxidative DNA damage. The simultaneous increased oxidation of mtDNA and deficiency of DNA repair could enhance the lesion to mitochondrial genome, potentially causing neuronal damages (Mecocci et al., 2018).

5.3. Stress-oxidant biomarkers implicated in NDs

Several evidence support the implication of the pro-oxidation and antioxidant defence biomarkers influenced by O_3 listed in Table 1 in the aetiopathogenetic mechanisms of NDs. Even for NDs, we prevalently focused on those implicated in the Nrf2 signalling (in bold in Table 1).

5.3.2 Alzheimer's Disease

AD is characterized by progressive loss of cognitive and behavioral deterioration, which leads to the impairment of daily and routine activities. It is one of the most prevalent NDs

manifesting 45 million people worldwide. AD is characterized by the deposition of protein aggregates, extracellular amyloid plaques (Aβ), intracellular tau (τ) or neurofibrillary tangles, and loss of synaptic connections in specific regions of brain (Schipper, 2010, Mattson, 2004, Selkoe, 2001). The neuropathological diagnostic feature of AD is the accumulation of neurotoxic Aβ oligomer peptides, which, along with τ protein, mediates neurodegeneration, thus causing neuroinflammation, impairment in synaptic connection, cholinergic denervation, neurotransmitter imbalance, neuronal loss, and dendritic alterations.

Different studies indicate the relationship between Aβ-induced oxidative imbalance and elevated levels of by-products of lipid peroxidation (e.g., 4HNE, MDA), protein oxidation (e.g., carbonyl), and DNA/RNA oxidation (e.g., OH8dG) (Wang, X. et al., 2014, Zhao, Y., Zhao, 2013, Pratico, 2008, Mecocci et al., 2018). These alterations were observed also in peripheral lymphocytes and lymphocyte mitochondria (for review (Mecocci et al., 2018). Higher levels of PC, measured in mitochondria extracted from lymphocytes, have been observed in AD (for review (Mecocci et al., 2018).

Decreased levels of antioxidant enzymes like SOD, CAT, GSH ~~and GSSG~~, decreased ratio of GSH/GSSG, and/or impaired expressions or activities of GSH-related enzymes have been observed in blood or brain of AD patients (Singh et al., 2019, Liu et al., 2004, Kim et al., 2006, Oliveira, Laurindo, 2018).

The RNS such as NO are also found to have a deleterious effect on neurons. Indeed, RNS elevation has been observed both in astrocytes as well as in neurons in an AD brain (for review (Singh et al., 2019). An increase in the expression of neuronal nNOS or NOS-1, cytokine-inducible iNOS or NOS-2, and endothelial eNOS or NOS-3 isozymes has been observed in AD astrocytes. The direct association of iNOS and eNOS with Aβ aggregates indicating towards beta amyloid assisted in the induction of NOS to produce NO, which in turn leads to the formation of 3-nitrotyrosine (NT) (Luth et al., 2002, Luth et al., 2001).

Other findings reported increased levels of CK2 in the hippocampus and temporal cortex of AD patients (Rosenberger et al., 2016) and increased levels in AOPP (Can et al., 2013, Altunoglu et al., 2015), compared to non-demented controls. It has been observed that AD patients show an increased oxidation of red blood cells GSH, which indicates oxidative stress in peripheral cells, and an increased level of plasma thiobarbituric acid reactive substances (TBARS), which indicates a higher free radical oxidation of plasma unsaturated phospholipids (Vina et al., 2005).

Moreover, HO-1 has been proposed as systemic marker in early sporadic AD (Schipper et al., 2000). Indeed, plasma HO-1 protein levels are significantly decreased in patients with probable sporadic AD (Schipper, 2007). The up-regulation of HO-1 in AD brain can be explained because of local oxidative stress. Instead, the mechanism responsible for the downregulation of HO-1 in the blood of AD patients remains unclear, even though the existence of a HO-1 suppressor that inhibits HO-1 mRNA levels in the lymphocytes in AD plasma has been proposed (Maes et al., 2006). However, the results about HO-1 plasma levels in patients with AD are controversial. A study founds no changes in the serum level of HO-1 in a big cohort of AD patients, as compared with elderly control subjects, whereas increased level were observed in PD patients, highlighting different mechanisms involved in the peripheral response to oxidative stress in the two diseases (Mateo et al., 2010). Moreover, another study reports that in plasma of probable AD patients, both HO-1 and biliverdin reductase (BVR) levels are increased because of the enhanced oxidative stress. The authors suggested that plasma BVR status, more than HO-1, can represent a potential biochemical marker for the prediction of AD at the earliest stages of disease (Di Domenico et al., 2012); for review (Nitti et al., 2018).

5.2 Parkinson's disease (PD)

PD is the second most prevalent neurodegenerative disorder, after AD, which is characterized by the progressive degeneration of the dopaminergic neurons located in the substantia nigra (SN) pars compacta (Spillantini et al., 1998) which affects movement. The main neuropathological hallmark of PD is the presence of intracellular inclusions known as Lewy bodies

(LBs) and neurites (LNs) (Forno, 1996); predominantly composed by misfolded and aggregated forms of the presynaptic protein α-synuclein (αSyn; a small protein with 140 amino acids abundant in presynaptic nerve terminals) (Spillantini et al., 1998). αSyn plays a role in synaptic transmission and dopamine levels adjustment. αSyn primarily affect tyrosine hydroxylase phosphorylation and activity and the expression level of dopamine transporter on the cell membrane.

Different evidence supported the involvement of the pro-oxidation and antioxidant defence biomarkers influenced by O_3 listed in Table 1 also with PD (focus on Nrf2). Altered levels of GSH and GSSG, decreased ratio of GSH/GSSG, and/or impaired expressions or activities of GSH-related enzymes have been detected in PD (Liu et al., 2004). TOS and OSI levels were found higher in the PD patients as compared to controls (Mota et al., 2019).

RNS also plays major role in nitrosative stress in PD. NO, produced by nNOS or iNOS was found in large quantities in cells, as well as in the extracellular space around dopaminergic neurons (Tieu et al., 2003). It has been observed that in PD brains, NO obstructs various enzymes including complex I and IV of the mitochondrial electron transport chain, hinders the function of proteins by forming S-nitrosothiols, mediates lipid peroxidation, resulting in elevated levels of ROS and brain deteriorating effect. *In situ* hybridization and immunohistochemical studies also established the role of NO in PD *via* postmortem brain tissue analysis, which indicates an elevated level of iNOS and nNOS in basal ganglia structures (Eve et al., 1998, Hunot et al., 1996). $ONOO^-$ has been shown to inhibit the presynaptic dopamine transporter, which mediates the uptake of dopamine from the synaptic cleft to stop dopamine signalling, and to refill the dopamine vesicles. Its inactivation will induce a decrease in dopamine delivery (Picon-Pages et al., 2019).

Oxidative damage in nucleic acids is likely to be a major risk factor for PD (Bosco et al., 2006, Puspita et al., 2017). Oxidative DNA lesions, such as 8-oxoguanine (8-oxoG), accumulate in nuclear and mitochondrial genomes during aging, and such accumulation can increase dramatically in these patients (Nakabeppu et al., 2007).

5.3 Amyotrophic Lateral Sclerosis (ALS)

Among the various neurodegenerative diseases, ALS is the most common type of motor neuron disease; it is sometimes called Lou Gehrig's disease, after the famous baseball player who had this condition. ALS is characterized by the progressive degeneration of upper and lower motor neurons in the spinal cord, cortex, and brainstem (Kikuchi et al., 2002). Although for most of the last 2 decades mutation of Cu–Zn SOD1 was the only genetic aberration associated with the onset of familial ALS, recent studies have discovered additional abnormalities associated with the onset of sporadic and non-SOD1 familial ALS. These include a host of RNA/DNA-binding proteins such as the 43-kDa transactive response (TAR) DNA-binding protein (TDP-43) and the fused in sarcoma/translocated in liposarcoma (FUS/TLS). The most common genetic mutation is identified as expanded GGGGCC hexanucleotide repeat in the non-coding region of the *C9Orf72* gene located on chromosome 9p21 (Mendez, Sattler, 2015).

Wang et al., (Wang, Z. et al., 2019) reported increased blood levels of 8-OHdG, MDA, and AOPP and decreased GSH and uric acid levels in the peripheral blood of ALS patients. These biomarkers have been found in sporadic ALS patient's urine, cerebrospinal fluid (CSF), blood, and individual tissues.

5.4 Huntington Disease (HD)

HD named after George Huntington in 1872, is a fatal and autosomal dominant inherited progressive neurodegenerative disorder, resulting in neuronal degeneration in the striatum followed by deterioration of the cerebral cortex and thalamus. HD is caused by a mutation in the *huntingtin* (*HTT*) gene. It is characterized by an abnormal extension in the cytosine–adenine–guanine (CAG) repeat in this gene, which in turn translates into an abnormally long repeat of polyglutathione in the mutant huntingtin protein. HD is mainly characterized by impaired motor and cognitive traits, personality change, and psychiatric illness (Vonsattel, DiFiglia, 1998).

Lipid peroxidation, DNA damage, and specifically protein carbonylation were found to be more pronounced in HD (Tunez et al., 2011). Dysregulation in cysteine metabolism was observed in HD (Paul et al., 2018). Cysteine plays vital roles in redox homeostasis, being a component of the

major antioxidant GSH and a potent antioxidant by itself. In HD patients, decreased GSH levels and increased lipid peroxidation was observed as compared with controls (Oliveira, Laurindo, 2018). In postmortem brain specimens of HD, a twofold increase of OH8dG in mtDNA was found in the parietal and slightly less in the frontal cortex compared to controls (Polidori et al., 1999).

6. Molecular mechanisms involving ozone (O_3), Nrf2 and vitagene network and their biological relevance in neuroprotection

At the core of adaptive responses at the cell and origin of biological organization is the concept of hormesis (Calabrese, V. et al., 2010). Hormesis describes a process that results in ameliorating and improve cellular stress resistance, survival, and longevity in response to sub-lethal levels of stress (Mattson, 2008). Generally, a favorable biological response to low exposure to any stressor is found within the hormetic zone, whereas cell damage occurs at higher doses. The hormetic dose response results from either a direct stimulation or through an overcompensation stimulatory response following disruption in homeostasis (Calabrese, E. J., Baldwin, 2000). This theory is, to date a frontier area of neurobiological research, focal to understanding and developing new/complementary therapeutic approaches to NDs. In this context, Nrf2 is considered as a hormetic-like pathway (Calabrese, V. et al., 2010).

It has widely been reported that the activation of Nrf2 by several different mechanisms (calorie restriction, physical exercise, polyphenols, mushrooms) can be a way to improve life health, due to its transcriptionally modulation on the vitagene network. Calabrese et al. (Calabrese, V. et al., 2010), performed an exhaustive review on this topic, and they described in detail each single element of the vitagene pathway. Members of the Hsp70s are, in their function as molecular chaperones, involved in folding of newly synthesized proteins and refolding of damaged or misfolded proteins, as well as in assembly and disassembly of protein complexes. Trx, is a major redox control system, consisting of a 12 kDa redox active protein Trx, and a homodimeric seleno-protein called TrxR1. TrxR1 is a flavoprotein that catalyzes the NADPH-dependent reduction of oxidized thioredoxin protein. It is usually located in the cytosol, but it translocates into the nucleus

in response to various stimuli associated with oxidative stress, thereby playing a central role in protecting against oxidative stress. Sirtuins are histone deacetylases which, in the presence of NAD^+ as a cofactor, catalyze the deacetylation reaction of histone substrates and transcriptional regulators. Sirtuins regulate different biological processes, such as apoptosis, cell differentiation, energy transduction, and glucose homeostasis.

Recent reviews support wide evidence on how different nutraceuticals/antioxidants can contrast aging and combat many associated pathologies, including NDs (Leri et al., 2020, Calabrese, E. J., 2020). Natural polyphenols (i.e. curcumin, resveratrol, flavonols present in *Ginkgo biloba* extracts, polyphenols present abundantly in the leaves and in the ripening fruits of the olive tree, *Olea europaea*), as well as mushrooms (*Hericium Erinaceus*, *Coriolus versicolor*) can significantly modulated Nrf2 and Nrf2-dependent vitagenes expression, showing neuroprotective action. This can potentially resolves pathologies such as AD, PD and also Meniere's Disease, another degenerative pathology (Amara et al., 2020, Trovato, Siracusa, Di Paola, Scuto, Fronte et al., 2016, Trovato, Siracusa, Di Paola, Scuto, Ontario et al., 2016, Trovato Salinaro et al., 2018, Scuto et al., 2019).

In line with these findings, several studies demonstrated that also O_3 can modulate the vitagene network expression. Pharmacologically, it acts in a hormetic fashion (Bocci, V. A. et al., 2011, Calabrese et al., 2013), according an inverted V shape curve. We researched studies for meta-analyses regarding Nrf2, HO-1, Hsp70, TrxR1 and sirtuins. Whereas no studies were performed between sirtuins, TrxR1 and O_3, the results indicated that O_3 can statistically increase the expression/protein levels of Nrf2, HO-1 and Hsp70 molecules (Figure 4, Random model, Z=4.72 $p<0.00001$ OR=1.80 95%CI:1.05-2.55, even after Bonferroni correction 0.05/3=0.016). Although our work has been excluded because we performed transcriptomic analyses (Scassellati et al., 2017), we confirmed the increase of the gene enconding HO-1 (*HMOX-1*), after difference concentrations of O_3. The high heterogeneity in effect size among the studies ($p<0.0001$ $I^2=66\%$) is essentially determined by two factors: different sources of samples (human, cell and animal models)

and different methodology (biochemical and western blot analyses, ultrastructural and immunocytochemistry evaluations) (supplementary material Table 1S). Where it was possible, we performed the analysis as homogeneously as possible: in this case, O_3 concentration (20μg/ml) and exposition time (max 24hr) were constant in all experimental conditions.

Interestingly, a study reported the benefit effect of O_3 on Menière's disease (Pawlak-Osinska et al., 2004). Moreover, as reported for polyphenols and mushrooms (Hsiao et al., 2016, Ferreiro et al., 2018, Oh et al., 2014, Pan et al., 2018, Hasanzadeh et al., 2020, Wang, Y. et al., 2019), O_3 has been found to be involved in β-catenin system (Emon et al., 2017) as well as in NLRP3 (nitrogen permease regulator-like 3) inflammasome (Yu et al., 2017, Wang, Z., Zhang et al., 2018).

All these evidence support that, as polyphenols and mushrooms, O_3 acts in the same direction. Induction of vitagenes after their supplementation/adminstration determines a maintained response to counteract intracellular pro-oxidant status, thus providing neuroprotection.

7. Effect of Ozone Oxidative Preconditioning on Oxidative Stress Injury

Preconditioning is a process whereby an initial low dose of a stressor agent upregulates adaptive mechanisms that enhance resilience against subsequent and acute stressor agents within a time-sensitive window of ~ 10–14 days. (Calabrese, E. J., 2016). Different studies demonstrated that the supplementation with *Coriolus versicolor* (Ferreiro et al., 2018, Scuto et al., 2019, Trovato Salinaro et al., 2018, Trovato, Siracusa, Di Paola, Scuto, Fronte et al., 2016), and *Hericium Herinaceus* (Trovato Salinaro et al., 2018, Trovato, Siracusa, Di Paola, Scuto, Ontario et al., 2016), biomass and polyphenols (Mao et al., 2019) can maintain the response to neutralize intracellular pro-oxidant/neuroinflammatory status, preventing different neurological conditions.

Same behaviour was also widely reported for O_3. The term "ozone oxidative preconditioning" (OzoneOP) was coined when repeated administration of O_3 at nontoxic doses facilitate adaptation to oxidative stress. This occurs through mild immune system activation, enhanced release of growth factors and/or activation of metabolic pathways that help maintain redox balance (increased SOD, GSH activities, decreased peroxidation).

The first studies on OzoneOP were conducted by Barber et al., 1999 (Barber et al., 1999) and Leon OS et al., 1998 (Leon et al., 1998). From 1998-1999, a plethora of investigations on this topic was conducted. In Table 2, we reported 65 findings, of which 55 on OzoneOP, whereas 10 are the studies were on postconditioning phenomenon.

We observed that OzoneOP exerts a protective effect on ischemia-reperfusion injury (IRI) in rat models of cochlear, hepatic, intestinal, renal, cardiac, lung and skeletal ischemia through an oxidative preconditioning mechanism that prevents the increase of the endogenous pro-oxidant and stimulates antioxidant mechanisms (Table 2). Some authors also developed an *in vitro* Hypoxia/Reoxygenation (H/R) model to simulate OzoneOP, using normal rat kidney epithelial (NRK-52E) cells. This to eliminate confounding variables linked to animal models (Wang, L., Chen, Liu, Chen, Weng, Qiu & Liu, 2014, Wang, L. et al., 2018). Interestingly, the results confirmed those obtained in *in vivo* animal model (Table 2).

OzoneOP prevents also other different kind of injury: lipopolysaccharide (LPS) injection, carbon tetrachloride, partial hepatectomy, total body irradiation, methotrexate, intraperitoneal injection of rat fecal material, sepsis, kidney and cardiac transplantation, contrast-induced nephropathy, induction of diabetes, cisplatin-induced nephrotoxicity, contrast-induced nephropathy agent, H_2O_2, doxorubicin, ototoxicity, noise exposure, hypothermia, lipofundin (Table 2).

Different methodological systems have been implemented in these studies. The several authors analysed differences in mRNA gene expression levels as well as protein levels in Western Blot and biochemical analyses. All authors performed morphological, histopathological, immunofluorescence, and immunohistochemistry evaluations, in parallel and in concordance with molecular investigations. Interestingly, in some cases, the effects observed were strongly dose and time-dependent (Table 2).

In some cases (10 in total), the studies have been performed in postconditioning, obtaining the same outcomes. León Fernández et al. (Leon Fernandez et al., 2012) investigated the systemic redox status of patients with low back pain and neck pain, and if O_3 oxidative postconditioning

modified the pathological oxidative stress and protected against oxidative protein damage. In 33 patients with diagnosis of disc hernia (DH), 100% showed a severe oxidative stress. Major changes in SOD, total hydroperoxides, AOPP, fructolysine, and MAD were observed. After O_3 postconditioning, there was a re-establishment of patients' cellular redox balance as well as a decrease in pain in both DH. This demonstrated that O_2-O_3 therapy protected against oxidation of proteins and reduced the pain.

8. Conclusions

According to (Cuadrado et al., 2018, Cuadrado et al., 2019), systems medicine identifies a cluster of chronic disease pathophenotypes including NDs in which Nrf2 plays a fundamental role. Similarly, Nfr2 is strongly implicated in aging processes (Zhang, H. et al., 2015, Schmidlin et al., 2019, Silva-Palacios et al., 2018). These condition/diseases share common mechanisms and results represent a first attempt to structure Nrf2 as a common therapeutic and systems medicine approach.

We here have presented extensively research and strength on the antioxidant activities of O_3 correlated with the interaction with Nrf2 (Galie et al., 2018, Siniscalco et al., 2018, Re et al., 2014, Vaillant et al., 2013), along with anti-apoptotic functions by acting on mitochondrial Bax, caspases, p53 and HIFα molecules (Yong et al., 2017, Guclu et al., 2016), pro-autophagy and bioenergetic activities on Kreb's cycle. This paper provides a road map for mechanism-based systems medicine where O_3-Nfr2-vitagene network play a crucial role in the modulation of the cellular redox balance, in the reduction of the formation of ROS/RNS, in the change of apoptotic and autophagy mechanisms (Vikram et al., 2017). This underlines the evidence to become potential new therapeutic targets for NDs, and at the sample time to reduce the aging physiological mechanisms and cognitive decline, potential risk factors to develop more severe neurodegeneration damage.

Challenges regarding treatments efficacy and costs still persist for NDs. Thus, we suggest that O_2-O_3 therapy could represent a useful, safe, no-invasive, no-pharmacological, economical, effective treatment for these neurodegenerative conditions. In the medical setting, this therapy employs a gas mixture of O_2/O_3, obtained from the modification of medical-grade O_2 using

certificated O_3 generator device (Bocci, V., 2011). Based on the basic mechanisms of action of O_3 in blood, the therapeutic range of O_3 has been precisely calculated and found to be 10–80 μg/ml of O_3 in blood (Schwartz-Tapia et al., 2015). O_3 medical preparations are classified into three types: ozonized water, ozonized oil and ozonized gas, whereas different and main routes of application with relative concentrations of O_3 are widely described in Schwart-Tapia et al., 2015 (Schwartz-Tapia et al., 2015).

The side effects are minimal; the World Federation of Ozone therapy (WFOT) estimates the incidence of complications at 0.0007%. Moreover, the treatment is not only perfectly tolerated but most of patients have reported a feeling of wellness and euphoria throughout the cycle. This fact explains why the compliance of the patients remains excellent throughout the years.

The mechanisms of the positive effects of O_3 are attributed not only to up-regulation of cellular antioxidant enzyme activity, but also to the activation of the immune and anti-inflammatory systems, modulation of NPRL3 inflammasoma, action on proteasome, enhancement in the release of growth factors from platelets, improvement in blood circulation and O_2 delivery to damaged tissues, and enhancement of general metabolism, along with being a potent bactericide, fungicide and virucidal with potential effect on gut microbiota (for review (Scassellati et al., 2020). Consequently, these combinatorial effects could impact on cognitive and neurodegenerative domains, directly or indirectly through the mediation of gut microbiota (Cattaneo et al., 2017). Nrf2-ARE and vitagene network, but also NF-κB (Nuclear Factor Kappa B Subunit 1), NFAT (nuclear factor activated T-cells), AP-1 (Activated Protein-1), HIFα are the principal signalling pathways on which O_3 exercises its effects (for review (Scassellati et al., 2020). These effects could be sharable with those involved in NDs, where high inflammation and oxidant state, mitochondria dysfunctions, metabolic alterations, and slowdown in regenerative processes and immune system characterize these disorders.

As reported in (Smith et al., 2017), to date systems are available and proposed to have a more precise measurement of the redox state of a patient. One system proposes simultaneously

measuring different biological markers in the blood such as GSH, GSH-Px, GST, SOD, CAT, conjugated dienes, total hydroperoxides, and TBARS. Using an algorithm, information can be gathered about the total antioxidant activity, total pro-oxidant activity, redox index, and grade of oxidative stress. Thus, systems like this can provide insights to the correct dosage and response to O_3 therapy based on oxidative stress levels seen in the patient.

With the awareness that further studies are needed, this review reports substantial scientific evidence for building a rationale of using the O_2-O_3 therapy for delay aging processes and neurodegeneration, exploiting well documented omni various functions of O_3. This therapy could represent a convenient, inexpensive monodomain intervention, working in absence of side effects that will permit to modulate the oxidant, but also immune, inflammatory, metabolic, microbiota and regenerative processes impaired in NDs.

There is a recent consistent upsurge of interest in complementary medicine, especially dietary supplements and foods functional in delaying the onset of age-associated NDs. O_3 along with other antioxidants (polyphenols, mushrooms) can open new neuroprotective strategies, and could represent therapeutic targets to minimize the deleterious consequences associated to oxidative stress, such as in brain aging and NDs.

Authors' Contributions

Catia Scassellati and Antonio Carlo Galoforo contributed equally to this work.

Declarations of interest: none

Conflicts of Interest

The authors have declared no conflict of interest.

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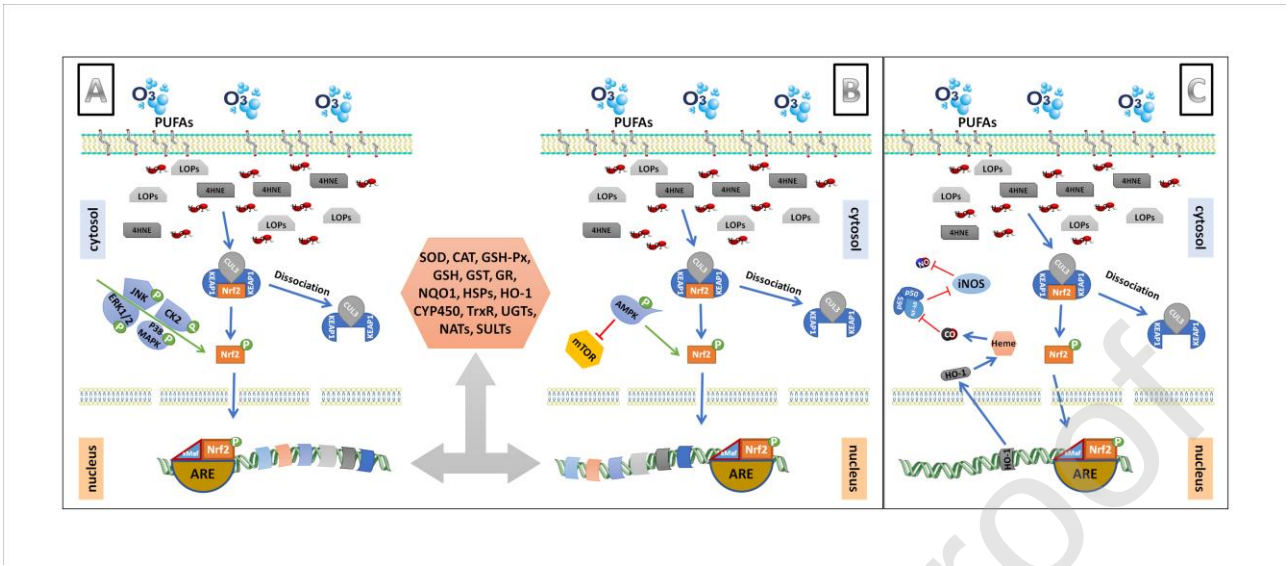
Figure 1. Molecular mechanisms linked to antioxidant/pro-authophagy activities of ozone (O_3) *via* Nfr2 signalling.



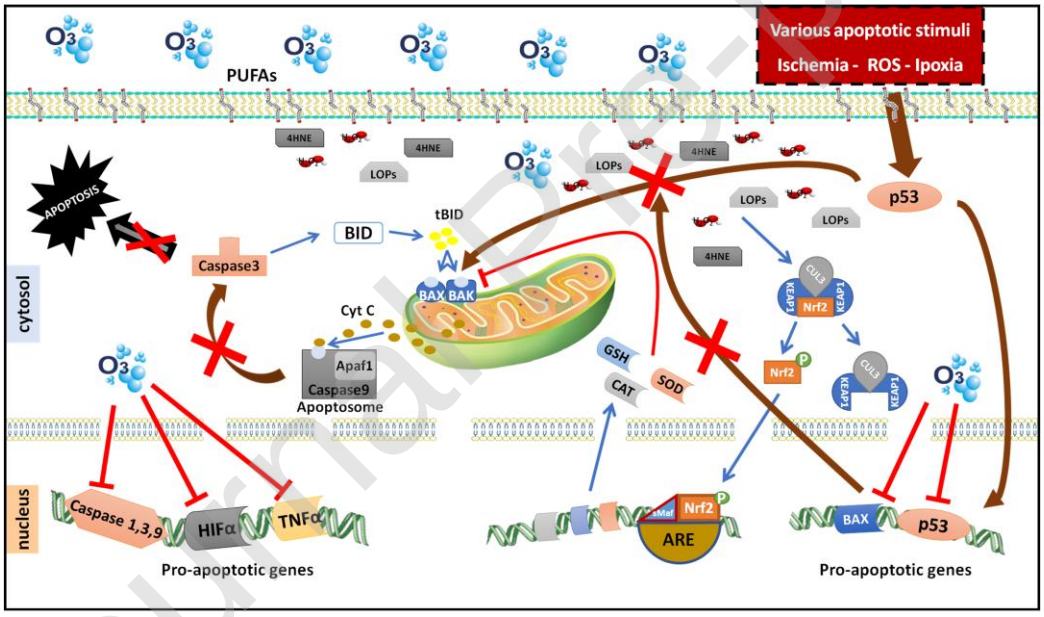
In the absence of stimuli, Nrf2 (nuclear factor erythroid 2–related factor 2) binds to its repressor Keap1 (kelch-like ECH-associated protein), an adapter between Nrf2 and Cullin 3 protein, which leads to ubiquitination followed by proteasome degradation. When O_3 is administrated, it dissolves immediately and it reacts with PUFA (Poly-Unsaturated Fatty Acids) leading to the formation of the two fundamental messengers: hydrogen peroxide (H_2O_2), 4-hydroxynonenal (4HNE) and lipid oxidation products (LOPs). These messengers can influence the modifications of cysteine residues present in Keap1 (S-HNE or—S—S) inhibiting ubiquitin conjugation to Nrf2 by the Keap1 complex and provoking the nuclear accumulation of Nrf2. Once in the nucleus, Nrf2 dimerizes and binds to cis-acting DNA AREs (Antioxidant Response Elements) in different genes: *Heme Oxygenase 1 (HO-1), Superoxide dismutases (SOD), Glutathione peroxidase (GSH-Px), Glutathione-S-Transferase (GST), Catalase (CAT), GSH-reductase (GR), NADPH quinone oxidoreductase 1 (NQO1), Heat Shock Proteins (HSPs), Cytochrome P450 monooxygenase, Thioredoxin reductase (TrxR), phase II enzymes (UDP-glucuronosyltransferases, UGTs; N-acetyltransferases, NATs, sulfotransferases, SULTs).*

A) O_3 involves casein kinase 2 (CK2), a regulator of the Nrf2 activity through its phosphorylation, and MAPK (mitogen-activated protein kinase) signalling pathway, that is inhibited with consequent inactivation of oxidative stress and apoptosis by O_3 administration.

B) O_3 modulates the degradation protein systems (autophagy), *via* activation of the AMP-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) signaling pathway.

C) O_3 downregulates inducible nitric oxide synthase (iNOS), which generates nitric oxide (NO) *via* NF-κB (Nuclear Factor Kappa B Subunit 1) pathway. (CO= carbon monoxide).

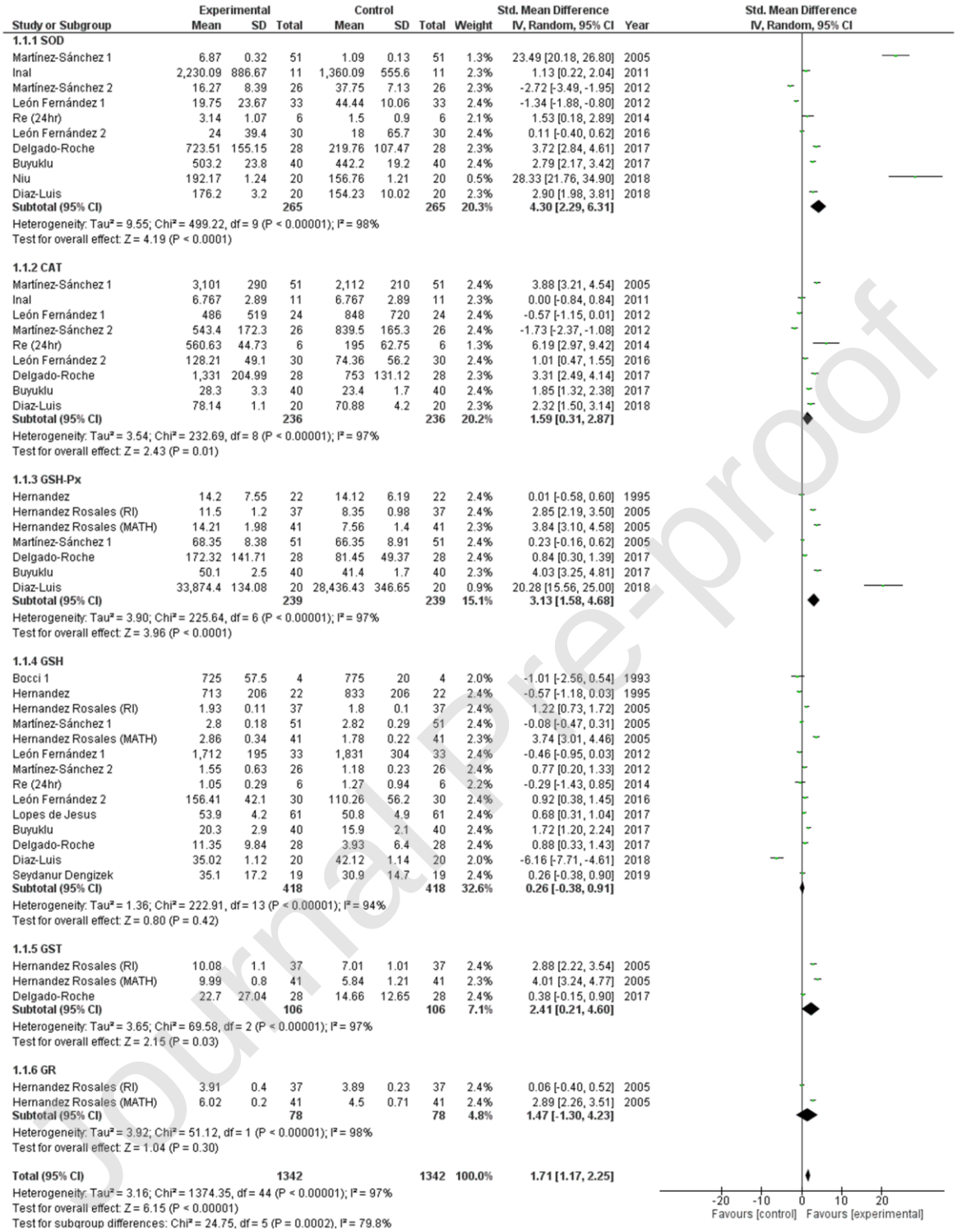
Figure 2. Molecular mechanisms linked to anti-apoptotic property of ozone (O_3) *via* pro-apoptotic molecules inactivation.



Various apoptotic stimuli (ischemia, reactive oxidant species, ROS, ipoxia) can activate directly p53 that in turn can play a role as transcription factor and activate the expression of pro-apoptotic genes. Among these, Bak (Bcl-2 homologous antagonist/killer) and Bax (Bcl-2-associated X protein) can stimulate in mitochondrial membrane the activation of Cytochrome C that in turn

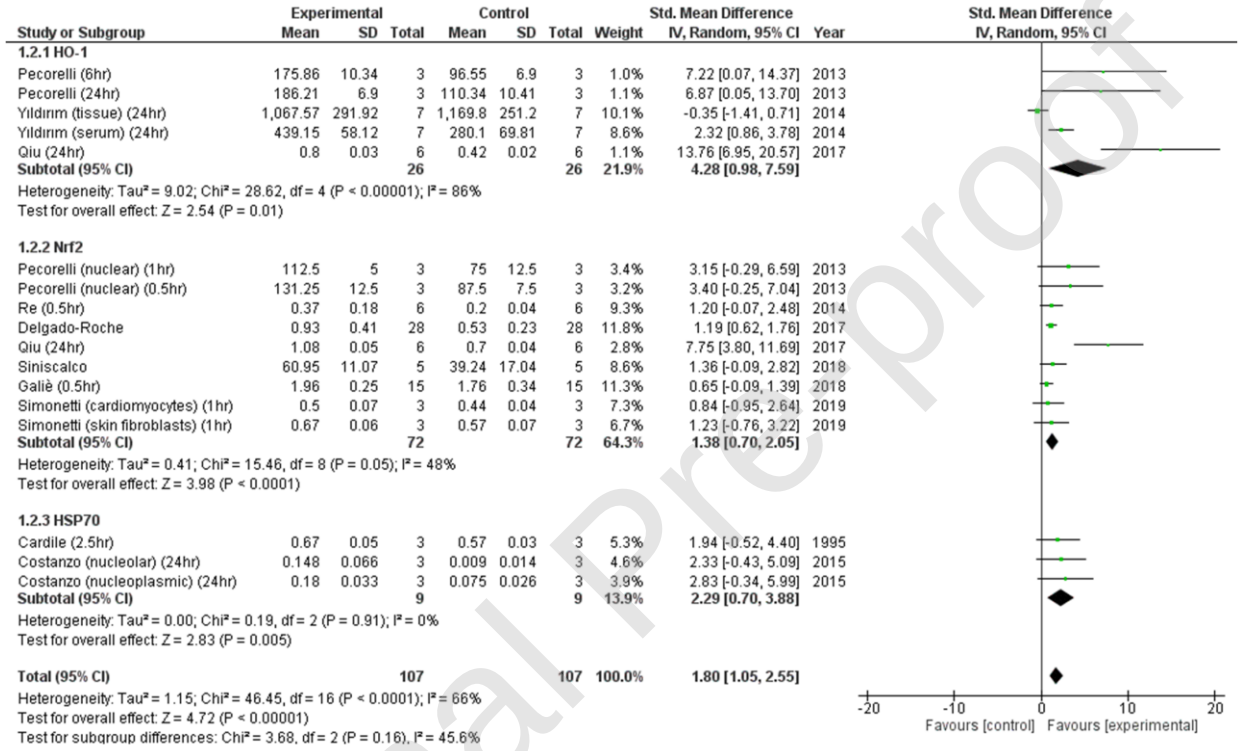
activates Apaf1 (Apoptotic protease activating factor-1) and caspase 9 to close the circle to stimulate the activity of caspase 3. Enzymes such as SOD (Superoxide dismutase), CAT (catalase), and GSH-Px (glutathione peroxidase), can regulate p53, Bax and Bcl-2. O_3 administration decreases the expression of *caspases 1-3-9, Hypoxia-inducible factor (HIFα), Tumor Necrosis Factor-α (TNF-α), Bax* and *p53* genes. (BID an acronym for BH3-interacting domain death agonist).

Figure 3 Forest plot for odds ratio from meta-analysis of the endogenous Nrf2- antioxidant pathway before and after ozone (O3) treatment.



CI, confidence interval; Chi^2, χ^2 test of goodness of fit; Tau^2, estimate of the between-study variance in a random-effects meta-analysis. Superoxide dismutase (SOD), catalase (CAT), Glutathione peroxidase (GSH-Px), Glutathione (GSH), Glutathione S-transferase (GST), Glutathione reductase (GR). RI= rectal insufflations; MATH=major autohemotherapy

Figure 4 Forest plot for odds ratio from meta-analysis of the endogenous Nrf2- vitagene pathway before and after ozone (O_3) treatment.



CI, confidence interval; Chi^2, χ^2 test of goodness of fit; Tau^2, estimate of the between-study variance in a random-effects meta-analysis. Nuclear factor Nrf2, heme-oxigenase (HO-1), heat shock protein (HSP)

Table 1. List of the pro-oxidation and antioxidant defence biomarkers influenced by ozone (O_3) and implicated in neurodegenerative disorders (NDs) as well as in aging processes.

Ozone biomarkers	Name and Function	Involvement in NDs	Involvement in Aging processes
4-HNE	4-Hydroxynonenal: a common aldehyde byproduct of lipid peroxidation during oxidative stress. 4-HNE is highly reactive and primarily produced in the brain via lipid peroxidation of arachidonic acid, a highly abundant omega-6 polyunsaturated fatty acids (PUFA) component of neuronal membranes. HNE may modify the ATP synthase, the final step in the production of ATP from electron transport chain (ETC) inside mitochondria. 4-HNE activates Nrf2 by alkylating thiol groups of cysteine residue in Keap1.	(Moldogazieva et al., 2019, Ayala et al., 2014, Baker et al., 2015)	(Benedetti et al., 2014, Csala et al., 2015)
8-OHdG	8-hydroxydeoxyguanosine (8-Oxo-2'-deoxyguanosine (8-oxo-dG): oxidized derivative of deoxyguanosine. Its concentrations within a cell are a measurement of oxidative stress (DNA oxidation). Reactive oxygen species (ROS) attack guanine bases in DNA easily and form 8-hydroxydeoxyguanosine, which can bind to thymidine rather than cytosine; thus, the level of 8-OHdG is generally regarded as a biomarker of mutagenesis consequent to oxidative stress.	(Wang, Z. et al., 2019, Nakabeppu et al., 2007, Poulsen et al., 2014, Polidori et al., 1999)	(Mecocci et al., 2018)
AOPP	Advanced Oxidation Protein Products: are group of oxidatively modified protein products containing dityrosine, pentosidine, and carbonyl-containing products generated by reactive oxygen species (ROS) or formed via myeloperoxidase reaction during oxidative/chlorine stress. They are biomarkers of oxidant-mediated protein damage	(Wang, Z. et al., 2019, Cristani et al., 2016)	(Maciejczyk et al., 2019, Cakatay et al., 2008, Komosinska-Vassev et al., 2012, Rusanova et al., 2018, Qing et al., 2012, Silva et al., 2015, Muller et al., 2015)
CAT	Catalase: it catalyzes the decomposition of hydrogen peroxide to water and oxygen. It is a scavenger enzyme of reactive oxygen species (ROS), protecting the cell from oxidative damage by ROS.	(Feitosa et al., 2018)	(Veal et al., 2018)
FRAP	Ferric Reducing the Ability of Plasma: total antioxidant capacity of plasma.	(Ademowo et al., 2017)	(Muller et al., 2015, Rizvi et al., 2006)
Fructolysine	It is an Amadori adduct of glucose to lysine. It is a precursor of the	-	-

	advanced oxidation protein products, which are induced by oxidative stress, and induces oxidative stress.		
GR	Glutathione reductase (or glutathione-disulfide reductase, GSR): it catalyses the reduction of glutathione disulfide (GSSG) to the sulfhydryl form glutathione (GSH), which is a critical molecule in resisting oxidative stress and maintaining the reducing environment of the cell.	(Feitosa et al., 2018, Liu et al., 2004, Rougemont et al., 2002)	(Veal et al., 2018)
GSH	Glutathione: it is antioxidant, capable of preventing damage to important cellular components caused by reactive oxygen species (ROS). It maintains cellular thiol status.	(Mazzetti et al., 2015, Liu et al., 2004, Gu et al., 2015, Rougemont et al., 2002, Oliveira, Laurindo, 2018)	(Maciejczyk et al., 2019, Teskey et al., 2018, Oliveira, Laurindo, 2018)
GSH-Px/GPx	Glutathione peroxidase: it has peroxidase activity whose main biological role is to protect the organism from oxidative damage. The biochemical function is to reduce lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water.	(Mazzetti et al., 2015, Gu et al., 2015, Rougemont et al., 2002)	(Maciejczyk et al., 2019, Veal et al., 2018)
GST	Glutathione S-transferase: it is phase II metabolic isozyme, known for the ability to catalyze the conjugation of the reduced form of glutathione (GSH) to xenobiotic substrates for the purpose of detoxification.	(Mazzetti et al., 2015, Gu et al., 2015, Rougemont et al., 2002)	(Veal et al., 2018)
HIF-1α	Hypoxia-inducible factor (HIF)-1alpha: is a subunit of a heterodimeric transcription factor hypoxia-inducible factor 1 (HIF-1). It is a basic helix-loop-helix PAS domain containing protein and is considered as the master transcriptional regulator of cellular and developmental response to hypoxia.	(Merelli et al., 2018)	(Yeo, 2019)
HO-1	heme-oxygenase-1: it catalyzes the conversion of heme into free iron, carbon monoxide and biliverdin. It possesses two well-characterized isoforms: HO-1 and HO-2. Under brain physiological conditions, the expression of HO-2 is constitutive, abundant and ubiquitous, whereas HO-1 mRNA and protein are restricted to small populations of neurons and neuroglia. HO-1 is an inducible enzyme that has been shown to participate as an essential defensive mechanism for neurons exposed to oxidant challenges, being related to antioxidant defenses in certain neuropathological conditions.	(Facchinetti, 2020)	(Schipper et al., 2019)
HSP70	Heat-Shock Protein 70: it is essential for the folding and repair of	(Lackie et al., 2017)	(Martinez de Toda, De

	damaged proteins. During stressful conditions, such as elevated temperature, it prevents protein aggregation, by facilitating the refolding or elimination of misfolded proteins. These mechanisms serve to promote cell survival conditions that would otherwise result in apoptosis.		la Fuente, 2015)
IMA	Ischemia-modified albumin: it measures ischemia in the blood vessels	(Altunoglu et al., 2015, Can et al., 2013)	-
LPO	Lipid peroxide: is the oxidative degradation of lipids.	(Feitosa et al., 2018, Negre-Salvayre et al., 2010)	(Negre-Salvayre et al., 2010)
MDA	Malondialdehyde: is a marker for oxidative stress. It is a reactive aldehyde produced by lipid peroxidation of polyunsaturated fatty acids.	(Feitosa et al., 2018, Wang, Z. et al., 2019, Ayala et al., 2014)	(Csala et al., 2015, Maciejczyk et al., 2019)
MPO	Myeloperoxidase: is a peroxidase enzyme. It requires heme as a cofactor. It is expressed in neutrophil and monocyte, and is implicated in various stages of inflammatory conditions with the production of a variety of potent oxidants.	(Ray, Katyal, 2016, Maki et al., 2019)	(Son et al., 2005)
Nfr2/CK2	Nuclear factor erythroid 2-related factor 2: is a basic leucine zipper (bZIP) protein that regulates the expression of antioxidant proteins that protect against oxidative damage triggered by injury and inflammation. Casein kinase 2: a serine/threonine-selective protein kinase implicated in cell cycle control, DNA repair, regulation of the circadian rhythm, and other cellular processes. Regulator of the Nrf2 activity through its phosphorylation.	(Perez et al., 2011, Sivandzade et al., 2019)	(Sivandzade et al., 2019)
NO	Nitric Oxide: is an important cellular signaling molecule which is derived from L-arginine by nitric oxide synthase (NOS). It works as a retrograde neurotransmitter in synapses, allows the brain blood flow, and has important roles in intracellular signaling in neurons from the regulation of the neuronal metabolic status to the dendritic spine growth. It is able to perform post-translational modifications in proteins by the S-nitrosylation of the thiol amino acids, which is a physiological mechanism to regulate protein function.	(Hannibal, 2016, Nakamura, Lipton, 2020, Radi, 2018)	(Picon-Pages et al., 2019)
NO-3/NO-2 (NOx)	Nitrate/nitrite: an index of NO production	(Hannibal, 2016, Nakamura, Lipton,	(Picon-Pages et al., 2019)

		2020, Radi, 2018)	
NOS	Nitric oxide synthase (inducible i II, endothelial e I): it catalyzes the production of nitric oxide (NO) from L-arginine.	(Hannibal, 2016, Nakamura, Lipton, 2020)	(Jung et al., 2012)
PCC/PCO	Protein carbonyl content: catalyses the carboxylation reaction of propionyl CoA in the mitochondrial matrix.	(Chevion et al., 2000, Fedorova et al., 2014)	(Cabiscol et al., 2014, Cakatay et al., 2008)
PP	Protein phosphatase: it is a serine/threonine phosphatase. It has been found to be important in the control of glycogen metabolism, muscle contraction, cell progression, neuronal activities, splicing of RNA, mitosis, cell division, apoptosis, protein synthesis, and regulation of membrane receptors and channels.	(Braithwaite et al., 2012, Clark, Ohlmeyer, 2019)	(Salminen et al., 2016)
SOD	superoxide dismutase: are the first and most important line of scavenger antioxidant enzyme defence systems against ROS and particularly superoxide anion radicals. There are two isoforms of SOD (cytoplasmatic CuZn- SOD or SOD1 and mitchondrial Mn- SOD or SOD2).	(Feitosa et al., 2018, Schaffert, Carter, 2020)	(Maciejczyk et al., 2019, Veal et al., 2018)
TAC	Total antioxidant capacity	(Mota et al., 2019)	(Maciejczyk et al., 2019)
TAS	Total antioxidant status	(Mota et al., 2019)	
TBARS	Thiobarbituric acid reactive substances: byproducts of lipid peroxidation (i.e. as degradation products of fats)	(Vina et al., 2005)	(Muller et al., 2015)
TH	Total Hydroperoxides: indicator of oxidative stress.	(Tarafdar, Pula, 2018)	
TOS	Total oxidant score	(Mota et al., 2019)	

Note: In bold the genes involved in Nrf2 signalling

Table 2. Preconditioning/postconditioning studies of O_3 on endogenous pro-antioxidant mechanisms in *vivo* on animal models and *in vitro* on cells.

Tissues	Dosages	Results	References
KIDNEY	Preconditioning: 0.7 mg/kg, intraperitoneally, 15 applications (once daily), before methotrexate (Mtx) (6 mg/kg).	Reduction: malondialdehyde (MDA). Increase: superoxide dismutase (SOD), glutathione peroxidase GSH-Px. Histologically: ILEUM: less inflammatory cell infiltration and edema, reduction in vacuolated cells in the epithelium; LIVER/KIDNEY: no significant change, due probably to the cumulative prolonged effect of Mtx on these tissues.	(Kesik et al., 2009)
	Postconditioning : **Sprague Dawley rats**: 1, 2 mg/kg, rectal insufflations, 15 applications, once a day, ischemia/reperfusion. **Renal tubular epithelial cell line, NRK-52E:** 20, 30, 40 μg/mL in complete medium, hypoxia–reoxygenation.	**IN VIVO**: Reduction dose-dependent manner: blood urea nitrogen (BUN), creatinine (Cr), malondialdehyde (MDA), bcl-2-associated X (BAX) and poly (ADP-ribose) polymerase 1 (PARP-1) expression, MAPK signaling pathway. Increase dose-dependent manner: superoxide dismutase (SOD). Histologically: ozone protected the tubular epithelium from swelling and from loss of the brush border. **IN VITRO:** Reduction dose-dependent manner: MAPK pathways, CREB, c-fos, bcl-2-associated X (BAX) and poly (ADP-ribose) polymerase 1 (PARP-1) expression, apoptosis, malondialdehyde (MDA), phosphorylation of p38, ERK1/2, and JNK. Increase dose-dependent manner: superoxide dismutase (SOD).	(Wang, L. et al., 2018)
	Postconditioning: **Sprague Dawley rats**: 2 mg/kg, rectal insufflations, 15 applications, once a day, after ischemia/reperfusion. **Renal tubular epithelial cell line, NRK-52E:** 20, 30, 40 μg/mL in complete medium, after hypoxia–reoxygenation.	**IN VIVO:** Reduction: blood urea nitrogen (BUN), creatinine (Cr), malondialdehyde (MDA), caspase 1, caspase 11, interleukin 1β (Il-1β), Interleukin-18 (IL18) expression/protein. Increase: superoxide dismutase (SOD). **IN VITRO**: Reduction: malondialdehyde (MDA), caspase 1, caspase 11, interleukin 1β (Il-1β), Interleukin-18 (IL-18) expression/protein. Increase: superoxide dismutase (SOD), cell viability. Histologic Examinations, Immunofluorescence Staining: prevented renal damage, reduction in Jablonski grading scale scores, decreased caspase 1.	(Wang, L. et al., 2019)
	Postconditioning: 0.5 mg/kg, rectal insufflation, after ischemia/reperfusion. A control with Oxigen was used.	Reduction: serum creatinine (Cr), blood urea nitrogen (BUN), myeloperoxidase (MPO), malondialdehyde (MDA), a-smooth muscle actin (α-SMA), transforming growth factor β1 (TGF-β1), phospho-Smad 2 protein. Increase: superoxide dismutase (SOD). Histology: Jablonski scores of histologic appearance in acute tubular necrosis, renal areas of tubulointerstitial fibrosis showed minimal phenomenon. Immunochemistry: Myofibroblasts (α-SMA positive) were faintly detected in ozone-treated samples.	(Jiang et al., 2020)
	Preconditioning: 1 mg/kg, rectal insufflations, 15 applications,	Reduction: α-smooth muscle actin (α-SMA), transforming growth factor-β1 (TGF-β1) expression/protein. Increase: Smad7 expression/protein.	(Wang, L., Chen, Liu, Chen, Weng,

	once a day, before ischemia/reperfusion.	Morphological/immunohistochemistry: increase in collagen staining, reduction in α-SMA expression.	Qiu, Liu & Zhu, 2014)
	Postconditioning: 0.5 mg/kg, daily for the 10 days' reperfusion, after ischaemia–reperfusion. A control was performed with Oxygen.	Reduction: serum creatinine (Cr), blood urea nitrogen (BUN), thiobarbituric acid reactive substances (TBARS). Increase: fructosamine, phospholipase A2, superoxide dismutase (SOD). Morphology: minimal alterations.	(Calunga et al., 2009)
	Preconditioning: 1 mg/kg, rectal insufflations, 15 applications, once a day, before the kidney transplantation.	Reduction: serum blood urea nitrogen (BUN), creatinine (Cr), malondialdehyde (MDA), renal allograft cell apoptosis index. Increase: superoxide dismutase (SOD), glutathione (GSH), catalase (CAT), nuclear factor erythroid 2-related factor 2 (Nrf-2), heme oxygenase 1 (HO-1). Morphological/immunohistochemistry: lower levels of damage, less severe renal allograft.	(Qiu et al., 2017)
	Preconditioning: 0.7 mg/kg/d, intraperitoneally, 5 days, before the induction of contrast-induced nephropathy. A control group was with Oxygen.	Reduction: serum blood urea nitrogen (BUN), creatinine (Cr), serum/renal malondialdehyde (MDA), total oxidant status (TOS). Increase: serum/renal nitric acid (NO), total antioxidant status (TAS). Histopathologic evaluation: reduction in degeneration of tubular epithelium, dilatation of Bowman capsule, necrosis in tubular epithelium, vascular congestion.	(Kurtoglu et al., 2015)
	Preconditioning: 1 mg/kg, rectal insufflations, 15 applications, once a day, before ischemia/reperfusion and/or ischemic preconditioning.	Reduction: malondialdehyde (MDA), urea nitrogen (BUN), creatinine (Cr), Jablonski grading scale scores. Increase: serum nitric acid (NO), NO synthase (endothelial, eNOS and inducible, iNOS) expression/protein, glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px). Histological Examination/Immunohistochemistry: improved renal dysfunction, histological damage, renal oxidative stress, increase presence of endothelial, eNOS and inducible, iNOS.	(Chen, Xing, Liu, Zhan, Zhou, Zhu & Chen, 2008b)
	Preconditioning: **IN VITRO Renal tubular epithelial cell line, NRK-52E,** 20, 30, 40 μg/mL in complete medium, before hypoxia/reoxygenation.	Reduction dose-dependent manner: 40 μg/mL apoptosis rate, malondialdehyde (MDA), Lactate dehydrogenase (LDH), bcl-2-associated X (BAX), Bcl2, poly (ADP-ribose) polymerase 1 (PARP-1) expression. Increase dose-dependent manner: superoxide dismutase (SOD). Immunocytochemistry: decrease in cleaved caspase3-positive	(Wang, L., Chen, Liu, Chen, Weng, Qiu & Liu, 2014)
	Preconditioning: 1 mg/kg, rectal insufflations, 15 applications, once a day, before ischemia/reperfusion.	Reduction: serum blood urea nitrogen (BUN), creatinine (Cr), malondialdehyde (MDA), myeloperoxidase (MPO), Tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), intercellular adhesion molecule (ICAM-1), monocyte chemoattractant protein 1 (MCP-1), Toll-Like Receptor (TLR4), nuclear factor (NF-κB) expression/protein, caspase-3, bcl-2-associated X (BAX), Bcl2. Morphology: decreased score in Jablonski scale histology grading.	(Chen, Xing B FAU - Liu, Xiuheng et al., 2008)
	Preconditioning: 1 mg/kg, rectal	Reduction: malondialdehyde (MDA), serum blood urea nitrogen (BUN), creatinine (Cr),	(Xing et al., 2015)

	insufflations, 15 applications, once a day, before ischemia/reperfusion.	tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), intercellular adhesion molecule (ICAM-1), monocyte chemoattractant protein 1 (MCP-1), Toll-Like Receptor (TLR4) and nuclear factor (NF-κB) expression/protein /immunoistochemical, caspase-3, bcl-2-associated X (BAX), Bcl2. Morphological/Immunoistochemical features: relieved tubular necrosis, medullary haemorrhage, congestion and development of proteinaceous casts, reduction in Jablonski scores.	
	Preconditioning: 1 mg/kg, rectal insufflations, 15 treatments, once a day, before ischemia/reperfusion. As control was used also Oxygen.	Reduction: serum blood urea nitrogen (BUN), creatinine (Cr), Jablonski grading scale scores, endothelin-1. Increase: serum nitric oxide (NO), NO synthase (endothelial, eNOS, inducible, iNOS) expression/protein, superoxide dismutase (SOD), glutathione (GSH), glutathione peroxidase (GSH-Px). Morphology: preservation of tissue histology.	(Chen, Xing, Liu, Zhan, Zhou, Zhu & Chen, 2008a)
	Postconditioning: 0.5 mg/kg, rectal insufflations, 10 applications, once a day, after ischemia/reperfusion. As control was used also Oxygen.	Histopathological/Morphology: no significant differences for filtration fraction and proteinuria, improvement in glomerular filtrate rate, renal plasma flow, creatinine, less overall histological damage.	(Fernandez Iglesias et al., 2011)
	Preconditioning: 1.1 mg/kg, intraperitoneal, 5 days, before induction of diabetes. Other groups were diabetic rats/insulin.	Reduction: Systolic blood pressure (SBP), Diastolic blood pressure (DBP), Glycosylated hemoglobin (HbA1c), serum blood urea nitrogen (BUN), creatinine (Cr), aldose reductase (AR), malondialdehyde (MDA). Increase: superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT).	(Morsy et al., 2010)
	Preconditioning: 25 mcg/ml, intraperitoneal, 15 days, before methotrexate (20 mg/kg).	Reduction: malondialdehyde (MAD), Myeloperoxidase (MPO), Tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β). Increase: glutathione (GSH). Histolopatologically: reduction in degeneration of glomerular structures, glomerular congestion, dilatation of Bowman's space, degeneration of proximal tubuli, degeneration of distal tubuli, tubular basal membrane wrinkling, vascular congestion, interstitial edema, inflammation and cell infiltration.	(Aslaner et al., 2015)
	Preconditioning: 0.36, 0.72, 1.1, 1.8, 2.5 mg/kg, rectal insufflations, 15 applications, before cisplatin-induced nephrotoxicity (6 mg/kg).	Reduction dose-dependent manner: creatinine (Cr) (0.72, 1.1 mg/kg), thiobarbituric acid-reactive substances (TBARS). Increase dose -dependent manner: glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) (0.72, 1.1 mg/kg), catalase (CAT). Histopathological changes: at doses of 1.8 and 2.5 mg/kg, histopathological significant improved changes in renal tissue	(Borrego et al., 2004)
	Preconditioning: 1 mg/kg, intraperitoneal, 6 hours before and 6 hours after contrast-induced nephropathy agent (10 ml/kg), 5	Increase: total antioxidant capacity (TAC), lipocalin (NGAL). No alteration in creatinine. Histopathological alterations: improving in Renal tubular injury, hemorrhage, cast formation.	(Ozturk et al., 2018)

	days.		
	Preconditioning: Major Ozonated Autohemotherapy in 5m blood rabbit, before ischemia/reperfusion.	Reduction: interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), white blood cells, neutrophil to lymphocyte ratio (NLR), ischemia-modified albumin (IMA), total oxidant status (TOS), oxidative stress index (OSI). Increase: total antioxidant status (TAS). Histopathological changes: reduced the tubular brush border loss (TBBL), tubular cast (TC), tubular necrosis (TN), intertubular hemorrhage congestion (IHC), dilatation of bowman space (DBS).	(Sancak et al., 2016)
	Preconditioning: 0.5 mg/kg, rectal insufflations, 15 treatments, before ischaemia/reperfusion. Oxygen was used as further control.	Reduction: Phospholipase A, Fructosamine. Increase: p-amino-hippurate (PAH), inulin, superoxide dismutase (SOD). Morphology: increased renal plasma flow (RPF), glomerular filtration rate (GFR).	(Barber et al., 1999)
	Preconditioning: 0.8, 2.4, 4 mg/kg, intraperitoneal, daily for 5 days, with/without sepsis. A control was performed with Oxygen.	Reduction: serum alanine amino transferase (ALT), aspartate amino transferase (AST), creatinine (CRE), thiobarbituric acid reactive substances (TBARS), myeloperoxidase (MPO). Increase: superoxide dismutase (SOD), glutathione peroxidase (GSH-Px).	(Rodriguez et al., 2009)
	Preconditioning: 1mg/kg, transrectal insufflations, once a day, 15 treatments, before the kidney transplant procedure.	Reduction: blood urea nitrogen (BUN), serum creatinine (Cr) (slightly), Jablonski grade, serum interleukin-6 (IL-6), IL-18, cyclooxygenase-2 (Cox-2), Malonaldehyde (MDA), nuclear factor NF-κBp65 and rabbit polyclonal anti-rat antibody (HMGB1) expression/protein. Increase: Superoxide Dismutase (SOD), Glutathione peroxidase (GSH-Px). Morphology: alleviated the morphological damages, attenuated the injury of brush border of proximal renal tubular, restrained the expression level of NF- κBp65 in renal tissue, suppressed the expression of HMGB1 in renal tissue.	(Wang, Z. et al., 2018)
	150 mg/kg, intraperitoneally, single dose for 10 days, at the same time *Escherichia coli* toxin (LPS) (20 mg/kg).	Reduction: lactate dehydrogenase (LDH) (Liver, Kidney, Lungs, Heart). Increase: Succinate Dehydrogenase (SDH) (Lungs, Heart), adenosine triphosphatase (ATPase) (no Kidney), acid phosphatase (AcPase) (Liver, Kidney, Lungs, Heart), β-Glucuronidase (Liver, Kidney, Lungs). Histochemically detected activity of succinate dehydrogenase (SDH): extinguished enzymatic activity in central parts of the lobule and paralleled by narrowing of zone I (Liver). Histochemically detected activity of lactate dehydrogenase (LDH): increased activity (hepatocytes, Kupffer cells, Liver). Histochemically detected activity of adenosine triphosphatase (ATPase): decrease intensity of the reaction for ATPase (Liver). Histochemically detected activity of acid phosphatase (AcPase): lower decrease in	(Madej et al., 2007)

		activity (Liver). Histochemically detectable activity of succinate dehydrogenase (SDH): the reaction in tubular epithelial cells was slightly more pronounced (Kidney). Histochemically detected activity of lactic dehydrogenase (LDH): less pronounced stimulation of enzyme in principal tubules and other portions of nephrons (Kidney). Histochemically detected activity of adenosine triphosphatase (ATPase): decreased intensity of the reaction in renal glomeruli and in walls of blood vessels, particularly those of low caliper (Kidney). Histochemically detected activity of acid phosphatase (AcPase): decreased intensity of the reaction pertained in principal tubuli and collecting duts (Kidney). Histochemically detected activity of succinate dehydrogenase (SDH): no more pronounced alterations (Lungs). Histochemically detected activity of lactate dehydrogenase (LDH): stimulation was less pronounced (Lungs). Histochemically detected activity of adenosine triphosphatase (ATPase): no changing (Lungs). Histochemically detected activity of acid phosphatase (AcPase): decreased activity (Lungs).	
LIVER	Preconditioning: 0.2, 0.4, 1.2 mg/kg intraperitoneally, once daily, for 5 days, before lipopolysaccharide (LPS) injection (30 mg/kg). Dexamethasone (30 mg/kg) used as a reference drug.	Reduction dose-dependent manner: thiobarbituric acid reactive substances (TBARS). Increase dose-dependent manner: glutathione peroxidase (GPx).	(Rodriguez et al., 2011)
	Preconditioning: 0.2, 0.4, 1.2 mg/kg intraperitoneally, once daily, for 5 days, before lipopolysaccharide (LPS) injection (0.1 mg/kg). Dexamethasone (30 mg/kg) used as a reference drug.	Reduction dose-dependent manner: serum Tumor Necrosis Factor (TNF)-alpha, thiobarbituric acid reactive substances (TBARS). Increase dose-dependent manner: glutathion-S transferase (GST), glutathione peroxidase (GSH-Px).	(Zamora et al., 2005)
	Preconditioning: 0.2, 0.4, 1.2 mg/kg, intraperitoneally, 0.2, 0.4 mg/kg, rectal application, once daily for five days, before lipopolysaccharide (LPS)	Reduction dose-dependent manner: serum Tumor Necrosis Factor (TNF)-alpha.	(Zamora et al., 2004)

	injection (0.1 mg/kg).		
	Preconditioning: 50 ug/ml (4.4–5.0 ml), 15 treatments, one per day, before carbon tetrachloride (CCl4). Ozone control groups were: 1. A control was with oxygen; 2. another control was ozone without CCl4.	Reduction: Aspartic alanine transaminase (AST), phospholipase A, hepatic lipid peroxidation (TBARS, thiobarbituric acid-reactive substances). Increase: cholinesterase (CHEase), superoxide dismutases (SODs), Catalase (CAT), Calcium-dependent (Ca-ATPase), gluthatione (GSH), glucose-6-phosphate dehydrogenase (G6PD). Morpho-metric evaluation of the hepatic damage: reduction of the damage area.	(Leon et al., 1998)
	Preconditioning: 1 mg/kg, rectal insufflation, 15 treatments, one per day, before ischaemia–reperfusion.	Reduction: Aspartic alanine transaminase (AST), serum alanine aminotransferase (ALT), malondialdehyde (MDA) + 4-hydroxyalkenals, nitrite/nitrate (NO_2^-/NO_3^-). Increase: superoxide dismutase (SOD), total hydroperoxide (TH), glutathione (GSH), Ratio GSH/GSSG.	(Ajamieh, H. H. et al., 2004)
	Preconditioning: 0.7 mg/kg, intraperitoneal, daily five times, before 70% partial hepatectomy.	Reduction: serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), tumor necrosis factor alpha (TNF-α). No alterations: interleukin-6 (IL-6). Histopathological examination: improve in liver weight, mitotic index, proliferating cell nuclear antigen (PCNA) labeling index.	(Gultekin, Cakmak et al., 2013)
	Preconditioning: 0.7 mg/kg, intraperitoneal, daily five times, before total body irradiation with a single dose of 6 Gy.	Reduction time-dependent manner: serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), tumor necrosis factor alpha (TNF-α), malondialdehyde (MDA). Increase time-dependent manner: superoxide dismutase (SOD). Histopathological examination: reduction in hepatocellular degeneration, inflammation, congestion and dilatation in both sinusoids and central veins; reduced inflammatory cell infiltrate in the lamina propria; regular villous structure, abundant goblet cells in the epithelium; reduced inflammatory cell infiltrate in the lamina propria.	(Gultekin, Bakkal et al., 2013)
	Preconditioning: 0.5 mg/kg, intraperitoneal, daily five times, before lipopolysaccharide (LPS) injection (20 mg/kg). Ketamine (5 mg/kg) used as a reference drug.	Reduction: Nuclear factor κB (NF-κB) staining. Morphology/Immunohistochemistry parameters: intact hepatic architecture, normal liver cell membrane integrity, little inflammatory cell infiltration (low NF-kB-positive staining).	(Sun, Pei, 2012)
	Preconditioning: 1 mg/kg, rectal insufflation, 15 treatments, one per day, before ischemia/reperfusion. Agonist (2-chloro N6 cyclo-pentyladenosine, CCPA), Antagonist (8-cyclopentyl-1,3-dipropylxanthine, DPCPX) of A1 subtype receptor.	Reduction: serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), nitric oxide (NO) (nitrite/nitrate (NO^-_2)/NO^-_3), adenosine deaminase (ADA), malondialdehyde (MAD), 4-hydroxyalkenals, attenuated GSSG increase, NF-kB (p65 subunit) expression, tumor necrosis factor alpha (TNF-α), heat shock protein-70 (HSP70). Increase: glutathione (GSH). Immunohistochemistry: remarkable preservation of the liver parenchyma architecture, prevention of the inflammatory recruitment.	(León Fernández et al., 2008)

	Preconditioning: 1 mg/kg, rectal insufflation, 15 treatments, one per day, before ischemia/reperfusion. Cycloheximide (CHX) to promote protein synthesis inhibition after OzoneOP treatment.	Reduction: serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), malondialdehyde (MAD), 4-hydroxyalkenals. Increase: SOD (MnSOD), glutathione (GSH), GSH/GSSG. Histological lesions: normal morphology of the acinus like sham-operated. Ultrastructural analysis: normal appearance of mithocondrial, rough endoplasmatic reticulum and peroxisome, no alteration on nucleus structure.	(Ajamieh, H. H. et al., 2005)
	Preconditioning: 1 mg/kg, rectal insufflation, 15 treatments, one per day, before ischemia/reperfusion and/or ischaemic preconditioning. Oxygen was another control comparison.	Reduction: serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), 5'-NT, malondialdehyde (MDA), 4 hydroxyalkenals. calcium, calpain, total Xanthine dehydrogenase (XDH), xanthine oxidase (XO). Increase: total sylfhydryl groups. Improvement in histological parameters: normal morphology of hepatic lobuli.	(Ajamieh, H. et al., 2002)
	Preconditioning: 1 mg/kg, rectal insufflation, 15 treatments, one per day, before carbon tetrachloride (CCl4) (1ml/kg). An ozone control group was ozone without CCl4.	Reduction: uric acid, lactate, thiobarbituric acid-reactive substances (TBARS). Increase: hepatic glycogen, liver weight (LW)/body weight (BW) ratios, superoxide dismutase (SOD), catalase (CAT). Histopathological findings: the permanence of glycogen deposits in hepatic cells was proved, only a minimal non-parenquimatous cell reaction co-existed around the central vein.	(Candelario-Jalil et al., 2001)
	Preconditioning: 0.7 mg/kg, intraperitoneal, 15 applications (once daily), before methotrexate (Mtx) (6 mg/kg).	Reduction: malondialdehyde (MDA). Increase: superoxide dismutase (SOD), glutathione peroxidase (GSH-Px). Histologically: ILEUM: less inflammatory cell infiltration and edema, reduction in vacuolated cells in the epithelium; LIVER/KIDNEY: no significant change, due probably to the cumulative prolonged effect of Mtx on these tissues.	(Kesik et al., 2009)
	Preconditioning: 10, 30, 50 μg/ml, intraperitoneal, 5 days, before sepsi induced by intraperitoneal injection of rat fecal material (0.5g per kg of animals weight) extracted from another donor rat. A control group was performed with Oxygen.	Reduction dose-dependent manner in LIVER/LUNG: conjugated dienes (CD), thiobarbituric acid-reactive substances (TBARS), Total pro-oxidant activity. Increase dose-dependent manner: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), Total antioxidant activity (TAC).	(Guanche et al., 2010)
	Preconditioning: 0.8, 2.4, 4 mg/kg, intraperitoneal, daily for 5 days, with/without sepsis. A control was with Oxygen.	Reduction: serum alanine amino transferase (ALT), aspartate amino transferase (AST), creatinine (CRE), thiobarbituric acid reactive substances (TBARS), myeloperoxidase (MPO). Increase: superoxide dismutase (SOD), glutathione peroxidase (GSH-Px).	(Rodriguez et al., 2009)

	150 mg/kg, intraperitoneally, single dose for 10 days, at the same time *Escherichia coli* toxin (LPS) (20 mg/kg).	Reduction: lactate dehydrogenase (LDH) (Liver, Kidney, Lungs, Heart). Increase: Succinate Dehydrogenase (SDH) (Lungs, Heart), adenosine triphosphatase (ATPase) (no Kidney), acid phosphatase (AcPase) (Liver, Kidney, Lungs, Heart), β-Glucuronidase (Liver, Kidney, Lungs). Histochemically detected activity of succinate dehydrogenase (SDH): extinguished enzymatic activity in central parts of the lobule and paralleled by narrowing of zone I (Liver). Histochemically detected activity of lactate dehydrogenase (LDH): increased activity (hepatocytes, Kupffer cells, Liver). Histochemically detected activity of adenosine triphosphatase (ATPase): decrease intensity of the reaction for ATPase (Liver). Histochemically detected activity of acid phosphatase (AcPase): lower decrease in activity (Liver). Histochemically detectable activity of succinate dehydrogenase (SDH): the reaction in tubular epithelial cells was slightly more pronounced (Kidney). Histochemically detected activity of lactic dehydrogenase (LDH): less pronounced stimulation of enzyme in principal tubules and other portions of nephrons (Kidney). Histochemically detected activity of adenosine triphosphatase (ATPase): decreased intensity of the reaction in renal glomeruli and in walls of blood vessels, particularly those of low caliper (Kidney). Histochemically detected activity of acid phosphatase (AcPase): decreased intensity of the reaction pertained in principal tubuli and collecting duts (Kidney). Histochemically detected activity of succinate dehydrogenase (SDH): no more pronounced alterations (Lungs). Histochemically detected activity of lactate dehydrogenase (LDH): stimulation was less pronounced (Lungs). Histochemically detected activity of adenosine triphosphatase (ATPase): no changing (Lungs). Histochemically detected activity of acid phosphatase (AcPase): decreased activity (Lungs),	(Madej et al., 2007)
LUNG	Preconditioning: 0.7 mg/kg, intraperitoneal, 5 applications (once daily), before total body irradiation (TBI) (6 Gy).	Reduction: malondialdehyde (MDA), serum tumor necrosis factor alpha (TNF-a), interleukin-1 beta (IL-1β). Increase: superoxide dismutase (SOD). Histopathological evaluation: reduction in alveolar area, interstitial congestion, and alveolar and bronchiolar hemorrhage.	(Bakkal et al., 2013)
	Preconditioning: 100 μg/kg, intraperitoneal, once daily for 10 days, before ischemia/reperfusion.	Reduction: malondialdehyde (MDA), myeloperoxidase (MPO), inflammasome (NLRP3), apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC), un-cleavable cysteine-requiring aspartate protease-1	(Wang, Z., Zhang et al., 2018)

	A control was performed with Oxygen.	(procaspase-1), cysteine-requiring aspartate protease-1 (caspase-1), apoptotic index, interleukin-1 beta (IL-1β). Increase: transcription factor Nrf2, superoxide dismutase (SOD). Macroscopic and histologic view: dark and edematous tissue, inter alveolar septum, rupturing and alveolar space hemorrhage disappear.	
	Preconditioning: 0.8, 2.4, 4 mg/kg, intraperitoneal, daily for 5 days, with/without sepsis. A control was performed with Oxygen.	Reduction: serum alanine amino transferase (ALT), aspartate amino transferase (AST), creatinine (CRE), thiobarbituric acid reactive substances (TBARS), myeloperoxidase (MPO). Increase: superoxide dismutase (SOD), glutathione peroxidase (GSH-Px).	(Rodriguez et al., 2009)
	Preconditioning: **IN VITRO A549 cell lines**, 1, 10, 20, 80 mol/L, before H_2O_2.	Reduction dose-dependent manner: bcl-2-associated X (BAX), nuclear factor NF-κβ, tumor necrosis factor alpha (TNF-α), Inducible nitric oxide synthase (iNOS), nitrite levels. Increase dose-dependent manner: catalase (CAT), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), glutathione (GSH) expression. Morphology: recovered the majority of cells from the toxicity, regenerated cell proliferation, prevented 9.6% and 11.0% of cell loss.	(Kucukgul et al., 2016)
	Preconditioning: 10, 30, 50 μg/ml, intraperitoneal, 5 days, before sepsi induced by intraperitoneal injection of rat fecal material (0.5g per kg of animals weight) extracted from another donor rat. A control group was performed with oxygen.	Reduction dose-dependent manner in LIVER/LUNG: conjugated dienes (CD), thiobarbituric acid-reactive substances (TBARS), Total pro-oxidant activity (TOS). Increase dose-dependent manner: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), Total antioxidant activity (TAC).	(Guanche et al., 2010)
	150 mg/kg, intraperitoneally, single dose for 10 days, at the same time *Escherichia coli* toxin (LPS) (20 mg/kg).	Reduction: lactate dehydrogenase (LDH) (Liver, Kidney, Lungs, Heart). Increase: Succinate Dehydrogenase (SDH) (Lungs, Heart), adenosine triphosphatase (ATPase) (no Kidney), acid phosphatase (AcPase) (Liver, Kidney, Lungs, Heart), β-Glucuronidase (Liver, Kidney, Lungs). Histochemically detected activity of succinate dehydrogenase (SDH): extinguished enzymatic activity in central parts of the lobule and paralleled by narrowing of zone I (Liver). Histochemically detected activity of lactate dehydrogenase (LDH): increased activity (hepatocytes, Kupffer cells, Liver). Histochemically detected activity of adenosine triphosphatase (ATPase): decrease intensity of the reaction for ATPase (Liver). Histochemically detected activity of acid phosphatase (AcPase): lower decrease in activity (Liver).	(Madej et al., 2007)

		Histochemically detectable activity of succinate dehydrogenase (SDH): the reaction in tubular epithelial cells was slightly more pronounced (Kidney). Histochemically detected activity of lactic dehydrogenase (LDH): less pronounced stimulation of enzyme in principal tubules and other portions of nephrons (Kidney). Histochemically detected activity of adenosine triphosphatase (ATPase): decreased intensity of the reaction in renal glomeruli and in walls of blood vessels, particularly those of low caliper (Kidney). Histochemically detected activity of acid phosphatase (AcPase): decreased intensity of the reaction pertained in principal tubuli and collecting duts (Kidney). Histochemically detected activity of succinate dehydrogenase (SDH): no more pronounced alterations (Lungs). Histochemically detected activity of lactate dehydrogenase (LDH): stimulation was less pronounced (Lungs). Histochemically detected activity of adenosine triphosphatase (ATPase): no changing (Lungs). Histochemically detected activity of acid phosphatase (AcPase): decreased activity (Lungs).	
HEART	Preconditioning: rectal insufflations as five applications per week. In a group: 0.3 mg/kg/day in the first week, and 0.5 mg/kg/day in the second week. In another group, 0.6 mg/kg/day in the first week, and 1 mg/kg/day in the second week, before ischemia/reperfusion. A group was performed with Oxygen.	Reduction dose-dependent manner: creatine kinase-MB (CK-MB), lactate, myeloperoxidase (MPO), total nitrate/nitrite (NOx), thiobarbituric acid reactive substances (TBARS). Increase dose dependent manner: Myocardial adenine nucleotides (ATP, ADP, AMP, TAN), glutathione (GSH). Histological examination, ultrastructural analyses: improvement in edema in between muscle fibers, and edema within muscle fibers, good myofibrillar arrangement with only slight edema around muscle fibers, mild mitochondrial swelling with decreased matrix density and mild disruption of mitochondrial cristae and vesiculation, slight margination of chromatin near nuclear membrane.	(Ahmed, L. A. et al., 2012)
	Preconditioning: 100μg/kg/day, intraperitoneally, once daily, 5 days, before ischemia/reperfusion. A control was performed with Oxygen.	Reduction: microtubule-associated protein 1 light chain 3 (LC3BI/II), PTEN-induced putative kinase 1 (PINK1), cytochrome c oxidase subunit IV (COX4), Caspase 3, myocardial apoptosis. Increase: nuclear factor (erythroid-derived 2)-like 2 (Nrf2), glutamate-cysteine ligase catalytic subunit (GCLC), glutamate-cysteine ligase modifier subunit (GCLM), superoxide dismutases (SODs) expression. Morphology: mild mitochondrial injury. Validation of: 1. nuclear extracts (TATA-binding protein (TBP) in nuclear extracts), 2. mitochondrial fractions separated from the cytoplasmic fraction (cytochrome c oxidase subunit IV (COX4) detectable).	(Meng et al., 2017)

	Preconditioning: 0.6 mg/kg, rectal insufflations, twice/week for the first 3 months, then once/week till the age of 15 months, in aged rats. A control was performed with Oxygen.	Reduction: malondialdehyde (MDA), protein carbonyls (Pr Co), lipofuscin, cytosolic Ca^{2+} (heart/hippocampus). Increase: glutathione (GSH), energy status (ATP, ADP) (heart/hippocampus), Na^{+}, K^{+}, ATPase (hippocampus).	(El-Sawalhi et al., 2013)
	Preconditioning: 50, 80 mL/kg, single (1x) or repetitive (5x) insufflation, in rat cardiac transplant model.	Prolonged cardiac allograft survival without any adjunctive immunosuppressive therapy, not alternated number of red blood cells, decreased number of thrombocytes, increase of white blood cells, mostly granulocytes.	(Stadlbauer et al., 2008)
	Preconditioning; 0.3 mg/kg, rectal insufflation, once on alternating days for 20 sessions, before doxorubicin (2 mg/kg). The oxygen group was a further control.	Reduction: pro- brain natriuretic peptide (BNP), malondialdehyde (MDA), advanced oxidation protein products (AOPP). Increase: superoxide dismutase (SOD), catalase (CAT). Morphology: slight damage, normal morphology of cardiac fibres. 90% survival rate, reduced loss of body weight.	(Delgado-Roche et al., 2014)
	150 mg/kg, intraperitoneally, single dose for 10 days, at the same time *Escherichia coli* toxin (LPS) (20 mg/kg).	Reduction: lactate dehydrogenase (LDH) (Liver, Kidney, Lungs, Heart). Increase: Succinate Dehydrogenase (SDH) (Lungs, Heart), adenosine triphosphatase (ATPase) (no Kidney), acid phosphatase (AcPase) (Liver, Kidney, Lungs, Heart), β-Glucuronidase (Liver, Kidney, Lungs). Histochemically detected activity of succinate dehydrogenase (SDH): extinguished enzymatic activity in central parts of the lobule and paralleled by narrowing of zone I (Liver). Histochemically detected activity of lactate dehydrogenase (LDH): increased activity (hepatocytes, Kupffer cells, Liver). Histochemically detected activity of adenosine triphosphatase (ATPase): decrease intensity of the reaction for ATPase (Liver). Histochemically detected activity of acid phosphatase (AcPase): lower decrease in activity (Liver). Histochemically detectable activity of succinate dehydrogenase (SDH): the reaction in tubular epithelial cells was slightly more pronounced (Kidney). Histochemically detected activity of lactic dehydrogenase (LDH): less pronounced stimulation of enzyme in principal tubules and other portions of nephrons (Kidney). Histochemically detected activity of adenosine triphosphatase (ATPase): decreased intensity of the reaction in renal glomeruli and in walls of blood vessels, particularly those of low caliper (Kidney). Histochemically detected activity of acid phosphatase (AcPase): decreased intensity of	(Madej et al., 2007)

		the reaction pertained in principal tubuli and collecting duts (Kidney). Histochemically detected activity of succinate dehydrogenase (SDH): no more pronounced alterations (Lungs). Histochemically detected activity of lactate dehydrogenase (LDH): stimulation was less pronounced (Lungs). Histochemically detected activity of adenosine triphosphatase (ATPase): no changing (Lungs). Histochemically detected activity of acid phosphatase (AcPase): decreased activity (Lungs).	
INTESTINE	Preconditioning: 0.7 mg/kg, intraperitoneal, daily five times, before irradiation of 500 cGy.	Reduction: malondialdehyde (MDA), myeloperoxidase (MPO). Increase: bursting pressure values of anastomosis, Hydroxyproline (HPO), superoxide dismutase (SOD). Histopathological evaluation: improving in anastomotic wound healing, granulation tissue development and histological changes corresponding to the local inflammatory response.	(Tasdoven et al., 2019)
	Preconditioning: 0.7 mg/kg, intraperitoneal, daily five times, before total body irradiation with a single dose of 6 Gy.	Reduction time-dependent manner: serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), tumor necrosis factor alpha (TNF-α), malondialdehyde (MDA). Increase: superoxide dismutase (SOD). Histopathological examination: reduction in hepatocellular degeneration, inflammation, congestion and dilatation in both sinusoids and central veins, reduced inflammatory cell infiltrate in the lamina propria, regular villous structure, abundant goblet cells in the epithelium, reduced inflammatory cell infiltrate in the lamina propria.	(Gultekin, Cakmak et al., 2013)
	Preconditioning: 0.7 mg/kg, intraperitoneal, 15 applications (once daily), before methotrexate (Mtx) (6 mg/kg).	Reduction: malondialdehyde (MDA). Increase: superoxide dismutase (SOD), glutathione peroxidase (GSH-Px). Histologically: ILEUM: less inflammatory cell infiltration and edema, reduction in vacuolated cells in the epithelium; LIVER/KIDNEY: no significant change, due probably to the cumulative prolonged effect of Mtx on these tissues.	(Kesik et al., 2009)
	Postconditioning: 0.7 mg/kg/day, intraperitoneally and intraluminally, laparotomy and/or ischemia/reperfusion.	Macroscopic Appearance: increase in mucosal weight in jejunum and ileum, bowel weight in jejunum, mucosal DNA and protein in jejunum and ileum, villus height and crypt depth in jejunum and ileum, crypt cell proliferation in jejunum and ileum, p-ERK protein. Reduction: Park's Injury Score in jejunum and ileum, enterocyte apoptosis in jejunum and ileum, caspase 3.	(Haj et al., 2014)
COCHLEAR	Preconditioning: 1 mg/kg, intraperitoneally, 7 days, before ischemia/reperfusion.	Reduction: apoptotic index, malondialdehyde (MDA), the total oxidant score (TOS). Increase: superoxide dismutase (SOD), glutathione peroxidase (GSH- Px), total antioxidant capacity (TAC), catalase (CAT). Histological evaluation: increased numbers of glial cells in the spiral ganglion, reduced level of vascularization.	(Onal et al., 2017)
	Postconditioning: 60 ug/mL,	Statistically significant differences in DPOAE results.	(Koçak et al., 2016)

	rectal and/or intratympanic, 7 days, after cisplatin-induced ototoxicity (5-mg/kg/day). The rats were tested with distortion product otoacoustic emissions (DPOAE).	Histopathological scoring: decreased stria vascularis damage, decreased inner–outer hair cell damage.	
	Postconditioning: 30 µg/ml, intravenous, daily administration for 14 days, at the same time with noise exposure.	Reduction: malondialdehyde (MDA), % mitochondrial swelling, mitochondrial membrane potential (MMP), Glutathione disulfide (GSSG), cytochrome c (Brain, cochlear). Increase: glutathione (GSH), glutathione peroxidase (GSH- Px), superoxide dismutase (SOD) (Brain, cochlear), ATP. Histopathological findings: prevents mitochondrial membrane potential (MMP) collapse, mitochondrial swelling, cytochrome c release.	(Nasezadeh et al., 2017)
SKELETAL	Preconditioning: 0.7 mg/kg, intraperitoneally; 4 doses, before ischemia.	Reduction: malondialdehyde (MDA), Serum nitrite-nitrate (NOx), Inducible nitric oxide synthase (iNOS) immunostaining. Increase: glutathione peroxidase (GSH- Px), superoxide dismutase (SOD).	(Koca et al., 2010)
	Preconditioning: 0.7 mg/kg, 6 days, before ischemic period and/or hypothermia.	Reduction: malondialdehyde (MDA), interleukin-1β (IL-1β), creatinine kinase (CK), aspartate aminotransferase (AST), K^+, nitric oxide (NO). Increase: glutathione peroxidase (GSH- Px), superoxide dismutase (SOD). iNOS immunohistochemical staining: mild intensity.	(Ozkan et al., 2015)
PANCREAS	Preconditioning: 50 µg/kg, intraperitoneally, once a day for seven days. Streptozotocin (STZ) (2ml). A control was performed with Oxygen.	Reduction: 4-hydroxynonenal (4-HNE), Poly(ADP-ribose) polymerase-1 (PARP-1), glucagon, glycemia. Increase: nuclear factor Nrf2, glutathione-s-transferase (GST), insulin, leptin. Immunohistochemistry: reduction in tissue degeneration evidenced by the partial restoration of normal cellular population size of islets of Langerhans and absence of islet damage. Immunofluorescence: reduction in cell death, decreased DNA damage.	(Siniscalco et al., 2018)
	Postconditioning: 0.7-mg/kg, intraperitoneally, daily for 3 days. induction of acute necrotizing pancreatitis. A control was performed with Oxygen.	Reduction: serum amylase, neopterin, lipase, aspartate aminotransferase (AST), alanine amino transferase (ALT), γ-Glutamyl transferase (GT), malondialdehyde (MAD). Increase: Alkaline phosphatase (AP), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD). Increase in weight. Lower number of infected rats. Histopathologic analyses: lower degrees of necrosis and leukocyte infiltration. Improving in the histological injury score.	(Uysal et al., 2010)
ARTHRITIS	Postconditioning: 80 mg/kg, articular space 3 times/week (3.5 weeks) after PG/PS-induced arthritis. A control was performed with Oxygen.	Reduction: TNFa and IL-1β expression/protein, nitric oxide (NO), Fructolysine. Increase: superoxide Dismutase (SOD), catalase (CAT). Ameliorate the join swelling, decrease of arthritis index. Histological results: normal morphology.	(Vaillant et al., 2013)

TESTICULAR	Preconditioning: 1mg/kg, intraperitoneally, before detorsion for 2 hours.	Reduction: Ischemia Modified Albumin (IMA), Total Oxidant Status (TOS), Oxidative Stress Index (OSI). Histopathological score: lower.	(Tusat et al., 2017)
OTHER	Preconditioning: 1 mg/kg, rectal insufflation, 15 sessions in 5 weeks, in alternated days, 2 mL/kg of lipofundin. A control group was performed with Oxygen.	Reduction: malondialdehyde (MDA), peroxidation potential (PP), advanced oxidation protein products (AOPP), nitric oxide (NO). Increase: glutathione (GSH). Histopathology: minimal lesions in the aortas, smaller intima/media ratio.	(Delgado-Roche et al., 2013)