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REVIEW ARTICLE

Ozone Therapy: Clinical and Basic Evidence of Its Therapeutic Potential

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Ozone has recently been subjected to criticism and emphasis in relation to clinical efficacy and toxicity, respectively. Without a doubt, ozone, in common with oxygen itself, is one of the most potent oxidants. Ozone is considered one of the major pollutants in urban areas. Nevertheless, increasingly widespread use lately has highlighted the potential benefits as a therapeutic agent when used according to well-defined and safe protocols. Basic studies conducted following rigorous scientific and ethical criteria have been proposed for scientific discussion. This paper concerns original data on an *in vivo* model of Parkinson's disease and published data on the effect of low ozone doses with any risk of toxicity excluded with the concentrations commonly used in medical applications.

Key Words: Ozone, Ozone preconditioning, Diabetes, Parkinson's disease, Rotenone, Ischemia/reperfusion.

Introduction

In recent years, emphasis and attention has been focused on the use of medical ozone. Despite ample clarification (1), confusion persists concerning its potential toxicity as an oxidant agent vs. the reported clinical efficacy. This confusion is a major factor preventing a more widespread acceptance.

Furthermore, the use in specialities so diverse as neurology, orthopedics, internal medicine, sports medicine, endocrinology, and others makes it difficult to categorize ozone as a therapeutic agent. This may cause conflicts between the different fields of application and the various medical areas.

Ozone is most commonly associated with intervertebral disc herniation (2) and only recently in the field of stomatology.

Regarding disc herniation and the so-called disc conflict, most ozone actions are bound to its peculiar effects on the biohumoral environment. The mistaken view of considering ozone as a simple mechanistic agent causing lysis and reduction of the herniated disc still persists.

The application of ozone in neuroradiology is considered scientific and successful based on the possibility of statistically estimating the reduction or disappearance of the anatomic protrusion.

In our opinion, this is not proof enough. A follow-up considering the status of the patient following treatment could better indicate the efficacy of the ozone treatment compared to different methodologies such as paravertebral, intradiscal, epidural, or intraforaminal injections. The huge variability of the clinical responsiveness of patients introduces further difficulties in the establishment of standardized studies. Recent advances in ozone effects and conditioning suggest a key role for trace elements. Studies are in progress to evaluate the ratio of Mn^{2+}, Cu^{2+}, Zn^{2+}, and other essential elements for the enzymatic activity of superoxide dismutase (SOD) and other enzymes involved in the ozone effect. Dietary insufficiency or impairment either in food supply or metabolic pathways may play a negative role in the efficacy of the ozone treatment.

Most recently published scientific data, partially ignored by some authors, are indicative of many basic effects of the ozone molecule and could form the basis for future

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randomized clinical studies. One of the aims of this study is to present the more recent data published in international journals.

The pharmacological theory first described by Erlich on drug action: *corpora non agunt nisi fixata*, intended as a drug-receptor interaction that, following the mass action law promotes and activates a function in the cell, is apparently not applicable to ozone. As pharmacologists and scientists, we are naturally drawn to the challenge of questioning the Erlich theory to demonstrate scientifically some pharmacological activities induced by a gas like ozone without a pure drug/receptor interaction.

In light of more recent pharmacological knowledge, we can consider ozone as a pro-drug which, at certain non-toxic doses, can induce a rearrangement of the biochemical pathways with the activation of a second messenger in a cascade with a multiple system action. Ischemic preconditioning represents the best similarity in this context.

Evidence that antioxidant enzymes, nitric oxide pathways, and other subcellular activities could be modulated by low ozone doses is now proven and could support the surprising effects of ozone in many pathological conditions.

Furthermore, in light of the study by Wentworth et al. (3), the scientific data reported here could be scientifically emphasized and pharmacologically indicative. Indeed, the authors demonstrate the physiological presence of an ozone-similar mediator during inflammation, indicating ozone as a new biomolecule with striking effects, which must be considered and studied following new strategies with newly constructed randomized-standardized clinical studies. Moreover, the mechanisms of action of ozone on blood biomolecules with the generation of several messengers responsible for biological effects have been well clarified since 2002 (4).

We report here some of the most relevant articles recently published on the effects induced by low ozone doses on various biochemical pathways linked to common and rare human pathologies.

Ozone and Diabetes

Basic Studies

Diabetes produces a large number of changes in vessels that affect the reactivity of smooth muscle and endothelium. Vascular endothelium appears to be a vulnerable target for hyperglycemia-induced metabolic changes. Activation of the polyol pathway, non-enzymatic glycosylation of proteins, and the increase of reactive oxygen species (ROS) play an important role in diabetes complications. Ozone has been used as a therapeutic agent and beneficial effects have been observed. However, so far only a few biochemical and pharmacodynamic mechanisms have been elucidated.

The possibility that ozone could induce a useful adaptation to chronic oxidative stress was described in 1996 (5).

Other studies (6–8) confirmed that controlled ozone administration may promote an oxidative preconditioning or adaptation to oxidative stress, preventing the damage induced by ROS.

Given that diabetes is a disorder associated with oxidative stress, it was postulated that ozone treatment might protect antioxidant systems and maintain at a physiological level other markers of endothelial cell damage associated with diabetic complications. A study using streptozotocin (STZ) as a diabetes inducer was designed to test the protective effect promoted by ozone. Ozone treatment improved glycemic control, increased aldose reductase, fructolysine content, advanced oxidation protein products, pancreas integrity, and prevented oxidative damage. Furthermore, increased nitrite and nitrate levels with respect to STZ group occurred, but without changes when compared to non-diabetic controls. The results of this study show that repeated administration of ozone in non-toxic doses might play a role in the control of diabetes and its complications (9).

In addition, ozone antioxidant properties preserved β-cell functions and reduced hyperglycemia. Together, these results suggest that this approach may represent a potential complement in the treatment of diabetes and its complications (10).

Clinical Studies

Because ozone therapy can activate the antioxidant system, influencing the level of glycemia and some markers of endothelial cell damage at a pre-clinical level, a study to investigate the therapeutic efficacy of ozone treatment in patients with type 2 diabetes and diabetic foot was done, aimed at comparing ozone efficacy with respect to antibiotic therapy. A randomized controlled clinical trial was performed with 101 patients divided into two groups: one (n = 52) treated with ozone (local and rectal insufflation of the gas) and the other (n = 49) treated with topical and systemic antibiotics. The efficacy of the treatments was evaluated by comparing the glycemic index, the area and perimeter of the lesions, the biochemical markers of oxidative stress, and the endothelial damage in both groups after 20 days of treatment. Ozone improved glycemic control, prevented oxidative stress, normalized levels of organic peroxides and activated superoxide dismutase. The pharmacodynamic effect of ozone in the treatment of patients with neuroinfectious diabetic foot can be ascribed to the possibility of its being a superoxide scavenger. Superoxide is considered a link between the four metabolic routes associated with diabetes pathology and its complications. Furthermore, healing of the lesions improved, resulting in fewer amputations than in the control group. There were no side effects. These results show that medical ozone treatment could be a complementary therapy in the treatment of diabetes and its complications (11).

Ozone and SOD

Many studies indicate that, after reoxygenation of the liver, oxygen free-radical formation may initiate the cascade of hepatocellular injury, necrosis/apoptosis, and subsequent infiltration of inflammatory cells. Although ROS can arise from a number of sources, xanthine oxidase (XO) is frequently implicated as a significant source of these toxic oxygen species. Ischemic preconditioning (IscheP) is an inducible and potent endogenous mechanism by which repeated episodes of brief ischemia/reperfusion (I/R) confer a state of protection against subsequent sustained I/R. On the other hand, it has been demonstrated that ozone at low doses is able to promote an oxidative preconditioning (OzoneOP) through the increase and preservation of antioxidant endogenous systems.

Superoxide is one of the most relevant radicals in biological regulation. Many regulatory effects are mediated by hydrogen peroxide and other ROS that are chemically derived from superoxide (12).

Although SOD could protect against liver I/R injury, administration of SOD does not protect the liver against I/R damage (13). The protein SOD degrades rapidly when administered parenterally. Gene delivery has been used to increase protein expression in the cell (14).

Although the benefit of ischemic preconditioning in the liver has already been suggested in a pilot clinical study (15), knowledge of the molecular mechanism is limited. Intermittent clamping currently is used in practice by many centers. Although the working of the protective mechanism of intermittent clamping still remains unclear, it has been assumed to be a similar mechanism to that described in ischemic preconditioning, mainly by reduction of apoptosis (16). A large number of pharmacological agents were shown to confer protection against ischemic injury in the liver. These agents include antioxidants, adenosine agonists and nitric oxide ($NO^{\cdot}$) donors. Nevertheless, only a few drugs are currently at the point of clinical application (15). However, not only adenosine production but also other mechanisms seem to be involved.

OzoneOP may promote a moderate oxidative stress which, in turn, increases antioxidant endogenous systems protecting against liver damage (6,17). The protective mechanism mediated by OzoneOP may involve protein synthesis. Elevated ROS concentrations induce gene expression in many cells, whose products exhibit antioxidative activity. A major mechanism of redox homeostasis is based on the ROS-mediated induction of redox-sensitive signal cascades that lead to increased expression of antioxidants (12).

To investigate the influence of the inhibition of protein synthesis on the protective actions conferred by OzoneOP in hepatic I/R, rats were treated with cycloheximide in order to inhibit protein synthesis before OzoneOP treatment. Plasma transaminases, malondialdehyde + 4-hydroxyalkenals (MDA + 4-HDA), and morphological characteristics were measured as an index of hepatocellular damage; Cu/Zn-SOD, Mn-SOD, catalase (CAT), total hydroperoxides (TH), and reduced glutathione (GSH) levels as markers of endogenous antioxidant system were evaluated. OzoneOP increased Mn-SOD isoform and ameliorated mitochondrial damage. Conversely, cycloheximide abrogated the protection conferred by OzonoOP and decreased Mn-SOD activity. Cellular redox balance disappeared when cycloheximide was introduced. Thus, protein synthesis is involved in the protective mechanisms mediated by OzoneOP, and ozone treatment preserved mitochondrial functions and cellular redox balance (18).

Ozone and Nitric Oxide

Liver transplantation is now accepted as the best treatment for end-stage liver disease. Nevertheless, hepatic I/R injury associated with liver transplantation and hepatic resections are unresolved problems in clinical practice. Many studies indicate that oxygen free-radical formation after reoxygenation of liver may initiate the cascade of hepatocellular injury. The effects of OzoneOP on NO generation and the cellular redox balance have been studied using the inhibitor of the NO synthesis N^{ω}-nitro-L-arginine methyl ester (L-NAME) (19). Indeed, a previous study reported the induction of NO by ozonated plasma (20). The following parameters were measured: plasma transaminases (aspartate aminotransferase, alanine aminotransferase) as an index of hepatocellular injury; in homogenates of hepatic tissue nitrate/nitrite levels and inducible nitric oxide synthase (iNOS) by immunohistochemistry as an index of $^{\cdot}NO$ production; SOD, CAT, and GSH levels as markers of the endogenous antioxidant system, and finally MDA + 4-HDA, TH and tumor necrosis factor (TNF-α) as indicators of oxidative stress. A correspondence between liver damage and the increase of $^{\cdot}NO$, CAT, TH, GSH, and MDA + 4HDA concentrations were observed along with a decrease of SOD activity. OzoneOP prevented and attenuated hepatic damage in OzoneOP +I/R and OzoneOP+L-NAME+I/R, respectively, in close relation with the above-mentioned parameters. Immunohistochemistry of iNOS showed that OzoneOP regulated enzymatic activity, whereas TNF-α levels were attenuated in the OzoneOP + I/R group. These results show that OzoneOP protected against liver I/R injury through mechanisms that promote a regulation of endogenous NO concentrations and the maintenance of cellular redox balance. Ozone treatment may have important clinical implications, particularly in view of the increasing hepatic transplantation programs.

There are different experimental results and opinions surrounding NO generation and its function in liver I/R injury as well as its protective effects. Nevertheless, the role of NO as a regulator of important processes in liver I/R is unquestionable.

Table 1. Effect of ozone preconditioning on cortical and striatal dopamine and norepinephrine content in rotenone-induced parkinsonism in rats ($n = 8$)

Parameters	Groups	Brain areas	
		Cortex	Striatum
Dopamine (μg/g tissue)	Normal control	0.625 ± 0.021†	7.813 ± 0.136†
	Rotenone	0.423 ± 0.017*	6.300 ± 0.260*
	Rotenone + ozone preconditioning	0.569 ± 0.021†	7.700 ± 0.110†
	Ozone	0.669 ± 0.031†	7.375 ± 0.280†
Norepinephrine (μg/g tissue)	Normal control	0.581 ± 0.031†	1.238 ± 0.057†
	Rotenone	0.419 ± 0.016*	0.688 ± 0.034*
	Rotenone + ozone preconditioning	0.560 ± 0.015†	1.091 ± 0.053*,†
	Ozone	0.605 ± 0.018†	1.163 ± 0.053 †

Data are mean ± SE.
*Significant difference from control group.
†Significant difference from rotenone group.
One-way ANOVA followed by LSD test, significant difference at $p < 0.05$.

OzoneOP regulated NO formation in the OzoneOP + I/R group and decreased the liver damage (increases in AST were prevented and those in ALT were attenuated). L-NAME was able to reduce NO generation in sham-operated + L-NAME and NO levels were not detectable in L-NAME + I/R group. Nevertheless, OzoneOP promoted NO formation in OzoneOP + L-NAME + I/R in spite of the presence of L-NAME, but less than OzoneOP + I/R. There was a concomitant increase in transaminase activities in this group (OzoneOP + L-NAME + I/R). These results suggest that the protection conferred by OzoneOP against the damage in liver I/R seems to be mediated, at least in part, by NO generation.

The contribution of OzoneOP to NO generation may be a consequence of its actions on gene expression. Punjabi et al. (21) and Pendino et al. (22) have shown that exposure to ozone causes NO production in macrophages and type II cells of rats, whereas Haddad et al. (23) demonstrated iNOS induction in rats. More recently, it has been found that ozone-induced lung hyperpermeability is associated with iNOS and that iNOS mRNA levels are mediated through Tlr-4, which has been identified as the gene that determines susceptibility to endotoxins. There was a correlative pattern of gene expression in two strains (ozone-susceptible and ozone-resistant, respectively), which support a role of Tlr4 in the regulation of iNOS during ozone exposure in the mouse (24).

Ozone and Parkinson's Disease

The aim of our work was the evaluation of OzoneOP on an *in vivo* model of rotenone-induced neurodegeneration in rats.

Oxidative stress has been implicated in numerous pathophysiological situations (25) being considered a unifying factor in the current theories of Parkinson's disease (PD) pathogenesis. This is because of the links between genetic and potential environmental factors in the onset and progression of the disease. Those environmental toxins that have the strongest association with PD phenotypes either cause high amounts of oxidative stress, such as rotenone, or directly increase the rate of α-synuclein aggregation, as with copper and other heavy metals (26). Furthermore, the aggregation of α-synuclein itself can cause oxidative stress (27) and oxidative stress can in turn cause conformational changes in α-synuclein (28). Even if the factors initiating the pathogenesis of PD and related neurodegenerative synucleinopathies are still largely unclear, many studies indicate a multiple brain mitochondria dysfunction after systemic treatment with pesticides or rotenone (29−31).

As mitochondrial oxidative damage plays an important role in the etiology of numerous oxidative stress-mediated clinical conditions, one possible protective strategy would be to enrich tissue mitochondria with antioxidants, thereby limiting mitochondrial oxidative damage, cellular injury and the initiation and progression of disease (32).

Controlled exposure to a physiologically low level of ROS can regulate a variety of key molecular mechanisms (33) and likely have a protective effect against subsequent exposure to severe oxidative stress. Recently, rotenone has given new clues to our current understanding of PD pathogenesis (34). Rotenone is a classical, high-affinity inhibitor of complex I, which has been widely used to

Table 2. Effect of ozone preconditioning on cortical and striatal nitric oxide content in rotenone-induced parkinsonism in rats ($n = 8$)

Groups	Basis areas	
	Cortex (nmol/g tissue)	Striatum (nmol/g tissue)
Normal control	44.250 ± 1.221†	52.375 ± 1.569†
Rotenone	66.500 ± 2.188*	70.500 ± 1.880*
Rotenone + ozone preconditioning	54.375 ± 1.475*,†	56.875 ± 1.315†
Ozone	51.750 ± 1.556†	54.125 ± 3.466†

Data are mean ± SE.
*Significant difference from control group.
†Significant difference from rotenone group.
One-way ANOVA followed by LSD test, significant difference at $p < 0.05$.

Table 3. Effect of ozone preconditioning on cortical and striatal oxidized and reduced glutathione content in rotenone-induced parkinsonism in rats ($n = 8$)

Parameters	Groups	Brain areas	
		Cortex	Striatum
Oxidized glutathione (μg/g tissue)	Normal control	2.097 ± 0.035†	2.130 ± 0.020†
	Rotenone	2.935 ± 0.048*	2.982 ± 0.029*
	Rotenone + ozone preconditioning	2.258 ± 0.035*,†	2.367 ± 0.030*,†
	Ozone	2.224 ± 0.027*,†	2.343 ± 0.023*,†
Reduced glutathione (μg/g tissue)	Normal control	2.306 ± 0.038†	2.343 ± 0.022†
	Rotenone	1.381 ± 0.039*	1.416 ± 0.042*
	Rotenone + ozone preconditioning	2.294 ± 0.036†	2.014 ± 0.043*,†
	Ozone	2.093 ± 0.034*,†	2.263 ± 0.079†

Data are mean ± SE.
*Significant difference from control group.
†Significant difference from rotenone group.
One-way ANOVA followed by LSD test, significant difference at $p < 0.05$.

understand the specific activity of the complex. Rotenone, being extremely lipophilic, freely crosses the blood-brain barrier and biological membranes, thus rapidly reaching the brain.

Repeated systemic exposure of rotenone has been reported to cause nigrostriatal dopaminergic degeneration in rats, producing an *in vivo* experimental model of PD (35).

The reductions in the activity of complex 1 of the mitochondrial electron transfer chain (ETC) may play an important role in rotenone-induced dopaminergic neurodegeneration in PD (36).

The irreversible inhibition of mitochondrial complex 1 results in energy depletion and increases mitochondrial oxyradical production to which dopaminergic neurons are vulnerable (37).

Materials and Methods

The duration of the study was 4 weeks and was conducted to evaluate the neurochemical effects of repeated exposure to rotenone in rats and to test the probable preventive effect of OzoneOP (as an indirect antioxidant) against rotenone-induced neurodegeneration in rats.

Sixty Sprague Dawley rats (Harlan, Indianapolis, IN) were included in the study, with an average weight of 175 g, divided into four groups: group I: control group, group II: ozone control group. Rats were given 5 mL of 25 μg/mL ozone in oxygen rectal insufflations (0.7 mg/kg). They received 20 sessions: five sessions per week for 4 weeks. In group III, rotenone was injected SC at a dose of 2 mg/kg/day every other day for a total of six injections during 11 days. Group IV was the study group (OzoneOP). Rats were given 5 mL of 25 μg/mL ozone in oxygen rectal insufflations (0.7 mg/kg). They received 20 sessions: five sessions per week for 4 weeks. After 10 sessions of ozone administration (2 weeks), rotenone was injected SC at a dose of 2 mg/kg/day every other day for a total of six injections during 11 days.

Groups of eight animals were sacrificed from each group, 24 h after the end of the study. Brains were quickly removed, washed with ice-cold saline, and the areas of cortex and striatum were isolated, weighed, and homogenized as 10% (w/v) either in 70% methanol (for dopamine and

Table 4. Effect of ozone preconditioning on cortical and striatal malondialdehyde (MDA) content in rotenone-induced parkinsonism in rats ($n = 8$)

Parameters	Groups	Brain areas	
		Cortex	Striatum
MDA (ng/g tissue)	Normal control	132.500 ± 3.273†	166.250 ± 3.504†
	Rotenone	266.375 ± 4.717*	250.125 ± 9.563*
	Rotenone + ozone preconditioning	166.875 ± 4.112*,†	178.750 ± 2.266†
	Ozone	154.375 ± 5.858*,†	176.250 ± 2.950†
Protein carbonyls (ng/mg protein)	Normal control	4.288 ± 0.191†	5.125 ± 0.183†
	Rotenone	6.163 ± 0.286*	7.000 ± 0.283*
	Rotenone + ozone preconditioning	4.838 ± 0.145*,†	5.713 ± 0.195*,†
	Ozone	4.588 ± 0.136†	5.375 ± 0.183†

Data are mean ± SE.
*Significant difference from control group.
†Significant difference from rotenone group.
One-way ANOVA followed by LSD test, significant difference at $p < 0.05$.

norepinephrine) or saline (remaining parameters). Preparation of cytosolic fraction was carried out by centrifuging an aliquot of tissue homogenate at 105,000 × g at 4°C for 45 min. The supernatant was used for the determination of SOD activity. Dopamine and norepinephrine were determined by the HPLC method according to Pagel et al. (38). Adenosine triphosphate determination was carried out according to the method of Zhang et al. (39). Determination of reduced and oxidized glutathione levels were carried out according to the methodology of Jayatilleke and Shaw (40). MDA levels were established according to Karatepe (41). Levels of reactive carbonyls in proteins were analyzed according to Levine et al. (42), modified by Adams et al. (43). NO (as nitrite and nitrate) was carried out according to the method of Everett et al. (44). Protein was determined according to Lowry et al. (45). Finally, histopathological examination was carried out according to Carleton and Drury (46).

One-way ANOVA followed by LSD test was used to evaluate significant differences from the controls.

Results

Rats were observed daily for the development of any signs of toxicity throughout the treatment period. Repeated rotenone treatment caused a marked decrease in both the food intake and the locomotor activity and induced muscle relaxation of both fore and hind limbs accompanied by high mortality rate in comparison to the other treatments.

Ozone therapy remarkably increased food intake and rate of weight gain compared to the control group and prevented mortality of the animals. Repeated treatment with rotenone significantly ($p < 0.05$) decreased the levels of dopamine and norepinephrine in both the cortex and striatum. OzoneOP significantly ($p < 0.05$) minimized the declining effect of rotenone on the levels of the two transmitters in the cortical and the striatal regions (Table 1).

In addition, rotenone treatment increased the level of NO, MDA, oxidized glutathione, and protein carbonyls of brain cortex and striatum. Rotenone-treated animals exhibited a significant ($p < 0.05$) decrease in the level of GSH, ATP, and depressed enzymatic activity of SOD (Tables 3–6).

OzoneOP significantly ($p < 0.05$) antagonized the disturbing effect of rotenone on the tested parameters in the cortical and the striatal regions (Tables 2–4).

Repeated rotenone treatment induced remarkable histopathological abnormalities in brain cortex, which are manifested as inflammatory, hemorrhagic, and neurodegenerative effects (Figure 2) in comparison to normal control (Figure 1).

OzoneOP remarkably attenuated the undesirable histopathological damage induced by rotenone (Figure 3). Ozone alone did not provoke any observable histological abnormalities (Figure 4).

Table 5. Effect of ozone preconditioning on cortical and striatal superoxide dismutase activity in rotenone-induced parkinsonism in rats ($n = 8$)

Groups	Brain areas	
	Cortex (μg/g tissue)	Striatum (μg/g tissue)
Normal control	13.875 ± 0.295†	14.250 ± 0.412†
Rotenone	12.375 ± 0.324*	12.363 ± 0.422*
Rotenone + ozone preconditioning	15.250 ± 0.452*,†	15.750 ± 0.366*,†
Ozone	17.375 ± 0.498*,†	17.125 ± 0.479*,†

Data are mean ± SE.
*Significant difference from control group.
†Significant difference from rotenone group.
One-way ANOVA followed by LSD test, significant difference at $p < 0.05$.

Rats treated with rotenone showed pathological changes manifested as karyolysis of nerve cell nuclei and perivascular edema (Figure 6) in comparison to normal midbrain architecture (Figure 5).

OzoneOP remarkably prevented the undesirable histopathological effects of rotenone (Figure 7). The histopathological feature of midbrain in ozone-alone-treated animals did not differ from the normal control group (Figure 8).

Discussion

The present biochemical and histopathological data might indicate the occurrence of cellular damage in the dopaminergic (DA) and noradrendergic (NE) neurons of both striatal and cortical areas by rotenone treatment. The concomitant decline in the levels of DA and NE mediators might indicate the inhibition of neurotransmitter synthesis through the inactivation of tyrosine hydroxylase (THY) and/or mitochondrial dysfunction and cell damage. THY may also exert unfavorable effects on DA and NE neurons

Table 6. Effect of ozone preconditioning on cortical and striatal adenosine triphosphate content in rotenone-induced parkinsonism in rats ($n = 8$)

Groups	Brain areas	
	Cortex (mmol/g tissue)	Striatum (mmol/g tissue)
Normal control	1.519 ± 0.028†	1.389 ± 0.013†
Rotenone	1.150 ± 0.029*	1.043 ± 0.024*
Rotenone + ozone preconditioning	1.550 ± 0.028†	1.329 ± 0.024†
Ozone	1.689 ± 0.019*,†	1.488 ± 0.018*,†

Data are mean ± SE.
*Significant difference from control group.
†Significant difference from rotenone group.
One-way ANOVA followed by LSD test, significant difference at $p < 0.05$.†

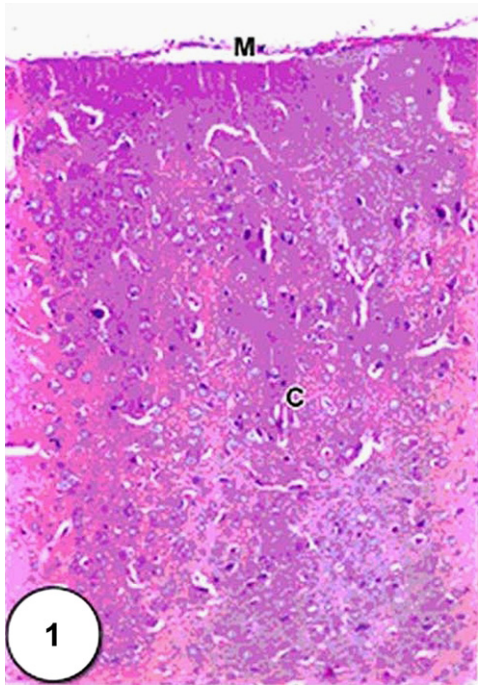

Figure 1. Transverse section of cerebral cortex of control rat showing the normal structure of the meninges (M) and cerebral cortex (C). H&E, ×40.

by generating ROS (47). Endogenously generated free radicals together with exogenous pro-oxidants have the ability to convert the parent catechols into their quinone derivatives, which can modify the structure of the THY enzyme

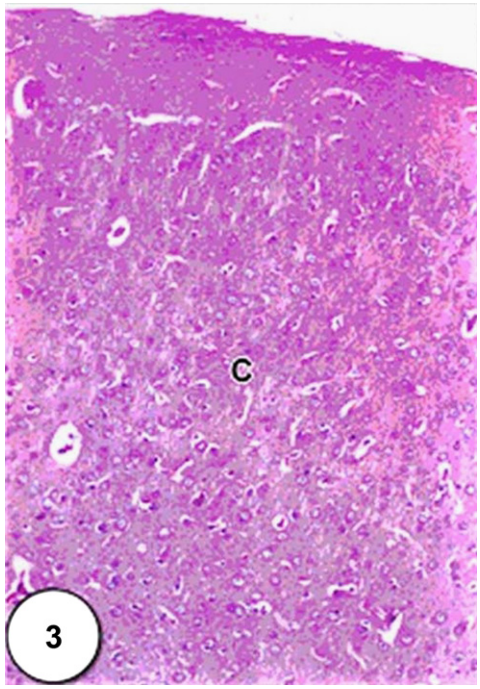

Figure 3. Transverse section of the cerebral cortex of rat injected with rotenone and ozone showing normal histological structure in the cerebral cortex and surrounding meninges. H&E, ×40.

leading to its inhibition (48). The depletion of ATP, GSH, and the increase of MDA and protein carbonyl levels might support the occurrence of oxidative stress resulting in lipid

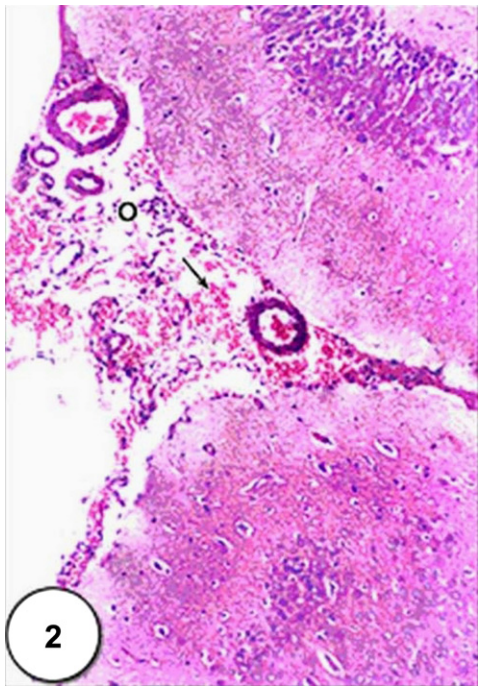

Figure 2. Transverse section of cerebral cortex of rat injected with rotenone showing severe hemorrhages (arrow), edema (o), and hyperemic blood vessels in the meninges covering the cerebral cortex. H&E, ×40.

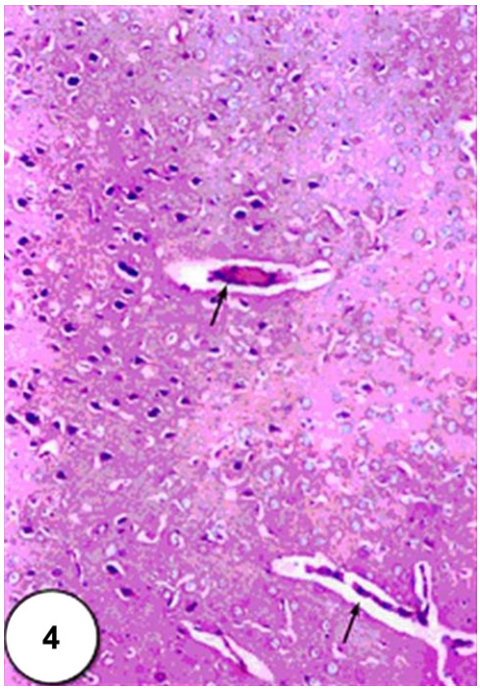

Figure 4. Transverse section of the cerebral cortex of rat injected with ozone showing perivascular edema surrounding the blood vessels (arrow). H&E, ×40.

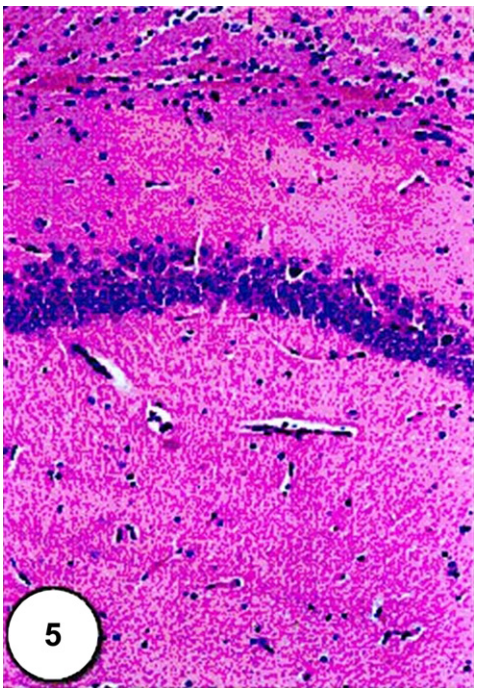

Figure 5. Photomicrograph of brain of control rat showing the normal structure of the midbrain. H&E, ×40.

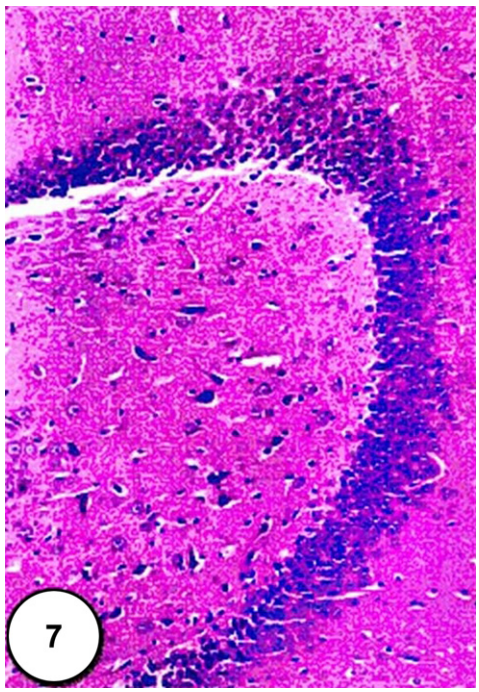

Figure 7. Photomicrograph of brain of rat treated with rotenone and ozone showing nearly normal structure of the midbrain. H&E, ×40.

and protein peroxidation and mitochondrial dysfunction, which lead to energy crisis and cell damage. In agreement with the biochemical data, histopathological findings indicated that rotenone-treated animals exhibited histological abnormalities in terms of degenerative changes and congested choroid plexi. Hemorrhage in focal areas and microvacuoles in the cytoplasm and pyknotic nuclei of cells of brain cortex and striatum were also observed.

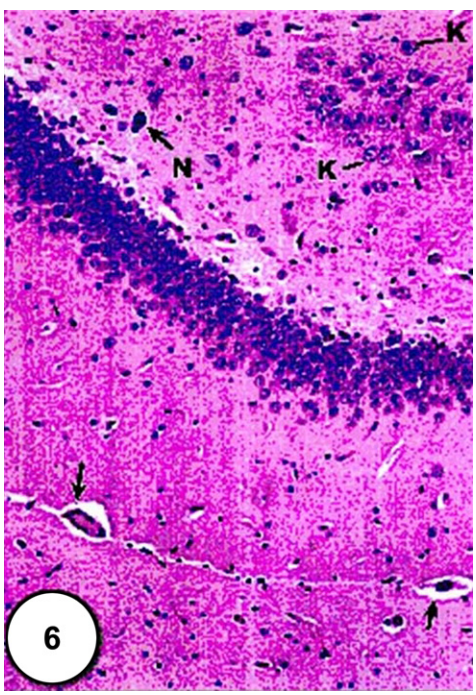

Figure 6. Photomicrograph of brain of rat treated with rotenone showing edematus perivascular changes (arrow). Nucleus of nerve cells exhibit signs of karyolysis (k) and necrosis (N) of the midbrain. H&E, ×40.

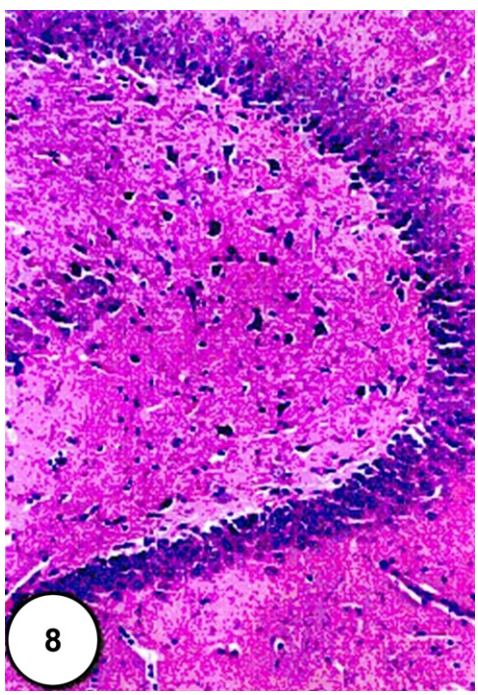

Figure 8. Photomicrograph of brain of rat treated with ozone showing the normal structure of the midbrain and no pathological cell changes. H&E, ×40.

Our study suggests that oxidative stress plays an essential role in rotenone toxicity and that OzoneOP may offer a remarkable protective effect against rotenone-induced brain toxicity.

Data presented in this paper are indicative of potentially positive effects induced by treatment with low doses of ozone. Particularly, an OzoneOP approach could be considered as a positive complement to the actual pharmacological therapies addressed to some pathologies such as diabetes and neurodegenerative disorders, promoting the regulation of endogenous NO concentrations and the maintenance of an adequate cellular redox balance.

References

1. Bocci V. Is it true that ozone is always toxic? The end of a dogma. Toxicol Appl Pharmacol 2006;216:493−504.
2. Andreula CF, Simonetti L, De Santis F, Agati R, Ricci R, Leonardi M. Minimally invasive oxygen-ozone therapy for lumbar disk herniation. AJNR Am J Neuroradiol 2003;24:996−1000.
3. Wentworth P Jr, McDunn JE, Wentworth AD, Takeuchi C, Nieva J, Jones T, et al. Evidence for antibody-catalyzed ozone formation in bacterial killing and inflammation. Science 2002;298:2195−2199.
4. Bocci V. Oxygen-Ozone Therapy: A Critical Evaluation. Dordrecht: Kluwer Academic Publishers;2002.
5. Bocci V. Does ozone therapy normalize the cellular redox balance? Implications for therapy of human immunodeficiency virus infection and several other diseases. Med Hypotheses 1996;46:150−154.
6. León OS, Menéndez S, Merino N, Castillo R, Sam S, Pérez L, et al. Ozone oxidative preconditioning: a protection against cellular damage by free radicals. Mediat Inflamm 1998;7:289−294.
7. Peralta C, León OS, Xaus C, Prats N, Candelario-Jalil E, Sala-Planell E, et al. Protective effect of ozone treatment on the injury associated with hepatic ischemia-reperfusion: antioxidant-prooxidant balance. Free Radic Res 1999;31:191−196.
8. Barber E, Menéndez S, León OS, Barber MO, Merino N, Calunga JL, et al. Prevention of renal injury after induction of ozone tolerance in rats submitted to warm ischaemia. Mediators Inflamm 1999;8:37−41.
9. Al-Dalain SM, Martínez-Sanchez G, Candelario-Jalil E, Menéndez S, Re L, Giuliani A, León OS. Ozone treatment reduces biomarkers of oxidative and endothelial damage in an experimental diabetes model in rats. Pharmacol Res 2001;44:391−396.
10. Martínez-Sanchez G, Al-Dalain SM, Menéndez S, Guiliani A, León OS. Ozone treatment reduces blood oxidative stress and pancreas damage in a streptozotocin-induced diabetes model in rats. Acta Farm Bonaerense 2005;24:491−497.
11. Martínez-Sanchez G, Al-Dalain SM, Menéndez S, Re L, Giuliani A, Candelario-Jalil E, et al. Therapeutic efficacy of ozone medical treatments in patients with diabetic foot. Eur J Pharmacol 2005;523:151−161.
12. Dröge W. Free radicals in the physiological control of cell function. Physiol Rev 2002;82:47−95.
13. Mizoe A, Kondo S, Azuma T, Fujioka H, Tanaka K, Hashida M, et al. Preventive effects of superoxide dismutase derivatives modified with monosaccharides on reperfusion injury in rat liver transplantation. J Surg Res 1997;73:160−165.
14. Prince HM. Gene transfer: a review of methods and applications. Pathology 1998;30:335−347.
15. Clavien PA, Yadav S, Sindram D, Bentley RC. Protective effects of ischemic preconditioning for liver resection performed under inflow occlusion in humans. Ann Surg 2000;232:155−162.
16. Rudiger HA, Kang KJ, Sindram D, Riehle HM, Clavien PA. Comparison of ischemic preconditioning and intermittent and continuous inflow occlusion in the liver. Ann Surg 2002;235:400−407.
17. Candelario-Jalil E, Mohammed-Al-Dalain S, Fernandez OS, Menendez S, Perez-Davison G, Merino N, et al. Oxidative preconditioning affords protection against carbon tetrachloride-induced glycogen depletion and oxidative stress in rats. J Appl Toxicol 2001;21:291−301.
18. Ajamieh HH, Berlanga J, Merino N, Martinez-Sánchez G, Carmona AM, Menendez-Cepero S, et al. Role of protein synthesis in the protection conferred by ozone-oxidative-preconditioning in hepatic ischaemia/reperfusion. Transplant Int 2005;18:604−612.
19. Ajamieh HH, Menéndez S, Martínez-Sánchez G, Candelario-Jalil E, Re L, Giuliani A, et al. Effects of ozone oxidative preconditioning on nitric oxide generation and cellular redox balance in a rat model of hepatic ischaemia reperfusion. Liver Int 2004;24:55−62.
20. Valacchi G, Bocci V. Studies on the biological effects of ozone: 11. Release of factors from human endothelial cells. Mediators Inflamm 2000;9:271−276.
21. Punjabi CJ, Laskin JD, Pendino KJ, Goller NL, Durham SK, Laskin DL. Production of nitric oxide by rat type II pneumocytes: increased expression of inducible nitric oxide synthase following inhalation of a pulmonary irritant. Am J Respir Cell Mol Biol 1994;11:165−172.
22. Pendino KJ, Gardner CR, Shuler RL, Laskin JD, Durham SK, Barton DS, et al. Inhibition of ozone-induced nitric oxide synthase expression in the lung by endotoxin. Am J Respir Cell Mol Biol 1996;14:516−525.
23. Haddad EB, Liu SF, Salmon M, Robichaud A, Barnes PJ, Chung KF. Expression of inducible nitric oxide synthase mRNA in Brown Norway rats exposed to ozone: effect of dexamethasone. Eur J Pharmacol 1995;293:287−290.
24. Kleeberger SR, Reddy SP, Zhang LY, Cho HY, Jedlicka AE. Toll-like receptor 4 mediates ozone-induced murine lung hyperpermeability via inducible nitric synthase. Am J Physiol Lung Cell Mol Physiol 2001;280:L326−L333.
25. Starkov AA, Fiskum G, Chinopoulos C, Lorenzo BJ, Browne SE, Patel MS, Beal MF. Mitochondrial α-ketoglutarate dehydrogenase complex generates reactive oxygen species. J Neurosci 2004;24:7779−7788.
26. Uversky VN, Li J, Fink AL. Metal-triggered structural transformations, aggregation, and fibrillation of human α-synuclein. A possible molecular NK between Parkinson's disease and heavy metal exposure. J Biol Chem 2001;276:44284−44296.
27. Turnbull S, Tabner BJ, El-Agnaf OM, Moore S, Davies Y, Allsop D. α-Synuclein implicated in Parkinson's disease catalyses the formation of hydrogen peroxide *in vitro*. Free Radic Biol Med 2001;30:1163−1170.
28. Ischiropoulos H. Oxidative modifications of α-synuclein. Ann NY Acad Sci 2003;991:93−100.
29. Norris EH, Uryu K, Leight S, Giasson BI, Trojanowski JQ, Lee VM. Pesticide exposure exacerbates alpha-synucleinopathy in an A53T transgenic mouse model. Am J Pathol 2007;170:658−666.
30. Panov A, Dikalov S, Shalbuyeva N, Taylor G, Sherer T, Greenamyre JT. Rotenone model of Parkinson disease: multiple brain mitochondria dysfunctions after short term systemic rotenone intoxication. J Biol Chem 2005;280:42026−42035.
31. Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, Greenamyre JT. Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nat Neurosci 2000;3:1301−1306.
32. Venditti P, De Rosa R, Portero-Otin M, Pamplona R, Di Meo S. Cold-induced hyperthyroidism produces oxidative damage in rat tissues and increases susceptibility to oxidants. Int J Biochem Cell Biol 2004;36:1319−1331.
33. Sen CK. Antioxidants and redox regulation of cellular signaling: introduction. Med Sci Sports Exerc 2001;33:368−370.

34. Ayala A, Venero JL, Cano J, Machado A. Mitochondrial toxins and neurodegenerative diseases. Front Biosci 2007;12:986−1007.
35. Sousa SC, Castilho RF. Protective effect of melatonin on rotenone plus Ca^{2+}-induced mitochondrial oxidative stress and PC12 cell death. Antioxid Redox Signal 2005;7:1110−1116.
36. Kweon GR, Marks JD, Krencik R, Leung EH, Schumacker PT, Hyland K, Kang UJ. Distinct mechanisms of neurodegeneration induced by chronic complex I inhibition in dopaminergic and non-dopaminergic cells. J Biol Chem 2004;279:51783−51792.
37. Jenner P. Parkinson's disease, pesticides, mitochondrial dysfunction. Trends Neurosci 2001;24:245−247.
38. Pagel P, Blome J, Wolf HU. High-performance liquid chromatographic separation and measurement of various biogenic compounds possibly involved in the pathomechanisms of Parkinson's disease. J Chromatogr B Biomed Sci Appl 2000;746:297−304.
39. Zhang YW, Long C, Shi SY. Determination of the adenosine triphosphate in myocardial tissue by ion-pair reversed-phase high performance liquid chromatography. Se Pu 2000;18:322−324.
40. Jayatilleke E, Shaw S. A high-performance liquid chromatographic assay for reduced and oxidized glutathione in biological samples. Anal Biochem 1993;214:452−457.
41. Karatepe M. Simultaneous determination of ascorbic acid and free malondialdehyde in human serum by HPLC-UV. LCGC NORTH AMERICA 2004;22:362−365.
42. Levine RL, Williams JA, Stadtman ER, Shacter E. Carbonyl assays for determination of oxidatively modified proteins. Methods Enzymol 1994;233:346−357.
43. Adams S, Green P, Claxton R, Simcox S, Williams MV, Walsh K, Leeuwenburgh C. Reactive carbonyl formation by oxidative and non-oxidative pathways. Front Biosci 2001;6:A17−A24.
44. Everett SA, Dennis MF, Tozer GM, Prise VE, Wardman P, Stratford MR. Nitric oxide in biological fluids: analysis of nitrite and nitrate by high-performance ion chromatography. J Chromatogr A 1995;706:437−442.
45. Lowry OH, Rosebrough NJ, Farr AL, Ramdall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193: 265−275.
46. Carleton HH, Drury RA. Histological Technique for Normal and Pathological Tissues and the Identification of Parasites. 5th ed. London: Oxford University Press;1973.
47. Haavik J, Almas B, Flatmark T. Generation of reactive oxygen species by tyrosine hydroxylase: a possible contribution to the degeneration of dopaminergic neurons? J Neurochem 1997;68:328−332.
48. Kuhn DM, Arthur RE Jr, Thomas DM, Elferink LA. Tyrosine hydroxylase is inactivated by catechol-quinones and converted to a redox-cycling quinoprotein. Possible relevance to Parkinson's Disease. J Neurochem 1999;73:1309−1317.