

Appraisal of ozone as biologically active molecule and experimental tool in biomedical sciences

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Received: 18 February 2010 / Accepted: 29 October 2010 / Published online: 11 November 2010
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Abstract Ozone (O₃) (CAS 10028-15-6) is a major air industrial pollutant and is well known for its very strong oxidative actions which affords the molecule its useful antimicrobial and deodorizing properties, but also its potential toxic effects. Knowledge of the activity and safety of ozone is important if its potential for use as a biologically active agent is to be realized, especially in view of the numerous unsubstantiated medicinal claims that are being made. To investigate ozone-induced oxidative stress as a model for investigating the neurobiology and treatment of certain central nervous system disorders, an experimental ozone inhalation model was developed to administer ozone to intact test animals following acute or chronic exposure. The model was successfully utilized to investigate the effect of dose and duration of exposure to ozone and its resultant effect on oxidative stress markers, depressive-like behaviours and response to antidepressant treatment. These studies demonstrate that the model not only is useful for studying the biological activity of ozone, but also for studying disorders of the brain associated with increased oxidative stress as well as the effects of altered redox status on drug treatment and response.

Keywords Ozone · Bioactivity · Toxicology · Oxidative stress · In vivo acute and chronic exposure · Experimental model

Introduction

Ozone (O₃), a highly reactive molecule and potent oxidant, is frequently used for chemical industrial applications replacing chlorine as a purifying and sterilizing agent for matrixes such as air, water and soil. In recent years there has been an ever increasing interest in ozone for its potential beneficial applications in biological systems, and in particular for various proposed medicinal applications. Ozone is a pale blue gas, slightly soluble in water and much more soluble in inert non-polar solvents, but much less stable than its diatomic allotrope (O₂). The resonance Lewis structures, electrostatic potential surface, space filling and ball-and-stick model of ozone are presented in Figs. 1 and 2. Ozone is a polar molecule with a C_{2v} symmetry (similar to the water molecule) as a bent structure with the O–O–O bond angle at 116.78°. Ozone exists as a bonding resonance hybrid with a single bond on one side and double bond on the other side, with an overall bond order for each side equals 1.5. The central oxygen atom is sp² hybridized with one lone pair. Ozone has dipole moment of 0.5337 D.

Various concerns have been raised in recent years regarding the safety and inappropriate medicinal use of ozone. This prompted us to conduct several investigations on the biological activity of ozone. Based on our own data as well as published information in the literature, the current appraisal briefly discusses the present status of ozone as a potential medicinal modality. Furthermore, considering the burgeoning interest in disorders characterized by increased oxidative stress, we also report on the development of an in vivo intact animal model with which to investigate the bio-behavioural effects of induced oxidative stress in rats following acute and/or chronic ozone exposure. These investigations can be performed in normal or

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Fig. 1 Resonance Lewis structures of the ozone molecule, with bond length and bond angle

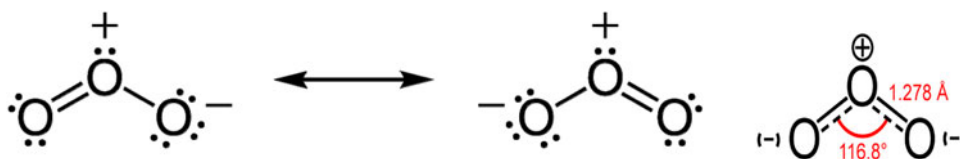
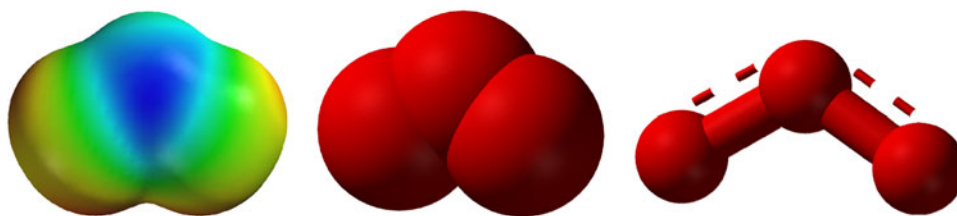


Fig. 2 Electrostatic potential surface, space filling and ball-and-stick model of ozone



pathologically challenged animals (e.g. stressed or genetic rodent models), and also to observe the interaction of oxidative stress with typical drug treatments used for the treatment of these disorders. Furthermore, this experimental model allows for investigations into the biological activity of ozone and ozone-derived reactive oxygen species (ROS—see below). Since some ROS are absorbed systemically and cross the blood brain barrier, this *in vivo* model ultimately allows for investigating the role of redox status in neuropsychiatric disorders as well as to study the actions of centrally active drugs under conditions of induced oxidative stress and/or altered brain redox status. This has particularly relevance for the treatment of neuropsychiatric disorders such as depression, but also central nervous disorders that are known to involve oxidative stress in their pathology. Indeed, there is an increased awareness of the role of environmental toxins in the development of mood disorders (Lupien *et al.*, 2009).

Appraisal: effects and applications of ozone

Adverse effects

Ozone is a major air pollutant that has been known for decades to cause toxic pulmonary effects in animals and man (Stokinger, 1965). Ozone, a strong oxidizing gas, is rapidly converted to ROS upon contact with cell proteins and lipids (Shelley *et al.*, 1989; Halliwell and Cross, 1994). Inhalation of ozone can induce rapid damage of epithelial cell membranes in the pulmonary airways. We earlier reported on the direct actions of ozone on isolated guinea pig tracheal chains resulting in potent contractions of this respiratory preparation, comparable to an acute bronchial asthma attack (Lotriet *et al.*, 2007) (see Fig. 3). These detrimental effects of ozone also involved an attenuation of the beneficial therapeutic effects of drugs clinically used in the treatment of asthma and potentiation of the bronchoconstrictory responses to allergenic agents. This study

cautioned against ozone exposure of patients with respiratory diseases. In addition, ozone has been demonstrated before to evoke bronchial constriction in healthy, non-smoking humans, supporting these findings (Kulle *et al.*, 1985). The risk to patients with airway diseases such as asthma, emphysema and chronic obstructive pulmonary disease is ever greater when exposed to inhaled ozone.

Biological adaptation and ozone pre-conditioning

Whilst exposure to ozone has been associated with cellular damage (Chuang *et al.*, 2009), including dose-dependent oxidative stress (Cross *et al.*, 1992), biological adaptation to these oxidative stressor effects may also occur (Stokinger, 1956), as has been observed in cultured cells (Chuang *et al.*, 2009), rodents (León *et al.*, 1998), plants Emberson *et al.*, 2007) and humans Hackney *et al.*, 1976). The mechanism of adaptation to ozone has not been fully elucidated, whilst recent studies in cultured cells indicated that the mitochondrial apoptotic pathway may play an important role in the mechanism of ozone-mediated adaptation (Chuang *et al.*, 2009).

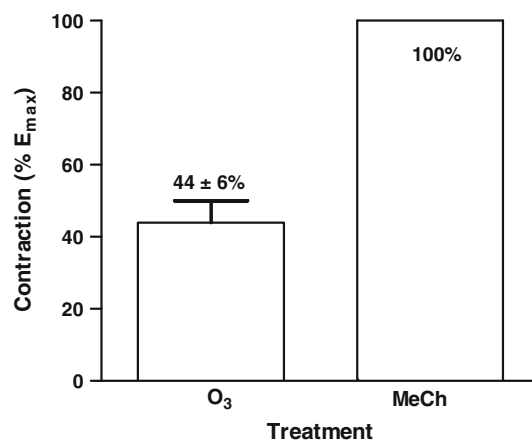


Fig. 3 Tracheal chain contraction of ozone (O₃) versus control methylcholine (MeCh) (adapted from Lotriet *et al.*, 2007)

We recently reported on the role of ozone in cellular adaptation proposing that both pro- and anti-apoptotic genes play a role in ozone-mediated adaptation to oxidative stress (Brink *et al.*, 2008). This investigation demonstrated that repetitive exposure to low doses of ozone, followed by a single high dose, results in the up-regulation of the genes encoding for Akt (protein kinase B), cAMP response element binding (CREB) protein and caspase 3. This process occurred over a period of 8 h. Whilst the up-regulation of the anti-apoptotic genes (Akt and CREB) may explain adaptation, it is not clear what the role or implication is for up-regulation of the pro-apoptotic gene, caspase 3. However, it is plausible that caspase 3 up-regulation may trigger a reactive up-regulation of various anti-apoptotic factors, leading to adaptation and improved cellular resilience (Brink *et al.*, 2008).

Several ozone-induced pre-conditioning studies have been conducted recently (Alvarez *et al.*, 2009; Rodríguez *et al.*, 2009; Stadlbauer *et al.*, 2008; Chen *et al.*, 2008a, b, c; Zamora *et al.*, 2004, 2005; Kesik *et al.*, 2009). It is clear from these studies that ozone treatment produces complex effects that are treatment regime-dependent with respect to time, dose and duration of exposure. The end result is, therefore, unpredictable, yielding either damaging or beneficial effects. This unpredictable response to redox-active agents is not unusual, as even antioxidants may have paradoxical effects on redox status dependent on the ambient redox milieu (Harvey *et al.*, 2008). A range of biological parameters are up- or down-regulated by the ozone treatments, such as the biological antioxidant systems, neurotransmitters, metabolites and cytokines. Several authors argue that the potential medicinal use of ozone could be linked to these pre-conditioning effects of ozone.

Ozone and the brain

In pre-clinical models, ozone-induced oxidation results in neurochemical and ultrastructural alterations both in peripheral and central nervous tissue. In the brain, some of the latter effects include an increase in lipid peroxidation leading to a decline in catecholamine turnover, as noted in the rat striatum and cortex, as well as an increase in basal dopamine, glutamate and nitric oxide and a decrease in serotonin (5-HT) (Rivas-Arancibia *et al.*, 2003). Furthermore, morphological changes have been found in the olfactory bulb and hippocampus in rats following 1–1.5 ppm acute ozone inhalation (Avila-Costa *et al.*, 1999; Colín-Barenque *et al.*, 1999). A number of dose-dependent behavioural changes in response to have been noted following the above-mentioned neurochemical changes, including alterations of the sleep–wake cycle (Paz and Huítrón-Reséndiz, 1996) and deterioration in long-term

memory and learning (Rivas-Arancibia *et al.*, 1998), as well as decreased motor activity (Avila-Costa *et al.*, 1999, 2001). Thus, ozone exposure has a marked effect on central nervous system function, including increased oxidative stress, changes in neurotransmitter turnover and various behavioural and cognitive changes. Despite evidence for its biological effects in the brain, few studies have investigated the effects of ozone administration on the efficacy of drug treatment on behaviour and brain neurochemistry.

Applications of ozone

In view of its powerful antimicrobial properties and the availability of ozone generators, ozone has found useful applications as an air (Kowalski *et al.*, 2003) and water disinfectant (Von Gunten, 2003a, b) in industry, in offices and in households. In the medical field, ozone is utilized in dentistry (Abu-Naba'a *et al.*, 2004; Azarpazhooh and Limeback, 2008; Holmes, 2003) and to treat wounds (Valacchi *et al.*, 2005; De Monte *et al.*, 2005), cancers (Jacobson *et al.*, 2005) and microbial infections. Means of ozone administration range from topical application to controversial autohaemotherapy (Di Paolo *et al.*, 2005; Travagli *et al.*, 2006; Cataldo and Gentilini, 2005). The latter involves withdrawal of a 50–200 ml sample of blood from the patient, mixing it with ozone and allowing it to react, and re-infusing back into the patient (Travagli *et al.*, 2006; Cataldo and Gentilini, 2005). Medicinal treatments with ozone (Bocci, 2006; Bocci *et al.*, 2009) and even unsubstantiated medicinal claims for the use of ozone to treat for example HIV-AIDS (Bocci, 1996), chronic ulcers (Bocci, 1996), retinitis pigmentosa (Werkmeister, 1969) and cardiac ischaemia (Bocci, 1996) are found in the literature. From the literature it is clear that ozone is increasingly being used for medicinal purposes in trial and error approaches without sufficient clinical data to support its safe and effective use. However, no medicines regulatory authority, such as the Food and Drug Administration of the USA, the European Medicines Agency of Europe and the Therapeutic Goods Administration of Australia, have to date approved any form of ozone treatment. It is, therefore, clear that our understanding of the biological actions, efficacy and safety of ozone is still insufficient to warrant its acceptance as a medicinal modality. Nevertheless, the wide range of biological activities of ozone warrants further study. Here a safe, reliable and accurate in vivo ozone exposure model, such as described in this article, may allow much needed high quality and robust research into the biological, pharmacological and environmental properties of ozone.

Ozone is currently administered to animals and humans as ozonated aqueous ointments, solutions and injections.

Routes of administration of ozone preparations include topical, oral, peritoneal, rectal, subcutaneous, intramuscular and intravenous.

Methodology: ozone inhalation model

Ozone is generally administered to animals and humans in the form of ozonated aqueous ointments, solutions and injections. Routes of administration of these preparations include topical, oral, peritoneal, rectal, subcutaneous, intramuscular and intravenous. In this study, we have developed a model of acute or chronic ozone exposure in rodents to investigate the extent of oxidative stress in the brain following ozone inhalation. Moreover, we also use the model to access the role of ozone-induced oxidative stress in the genesis of depressive-like behaviour in rats following a situational stressor (e.g. forced swimming) and its response to antidepressant treatment. Animals were exposed to acute (1 day) or chronic (30 days) controlled ozone inhalation (0, 0.25 and 0.7 ppm) and subsequently treated with an acute dose of vehicle or the antidepressant, imipramine (1 day). Subsequently, oxidative stress (measured as a function of superoxide free radical formation) was determined by measuring the amount of formed diformazan (nmol/mg protein), whilst membrane oxidative damage was determined by measuring the formation of malondialdehyde (nmol/mg protein). Both products were determined in the frontal cortex, one of the principle brain

regions involved in the behavioural and biochemical pathology of depression.

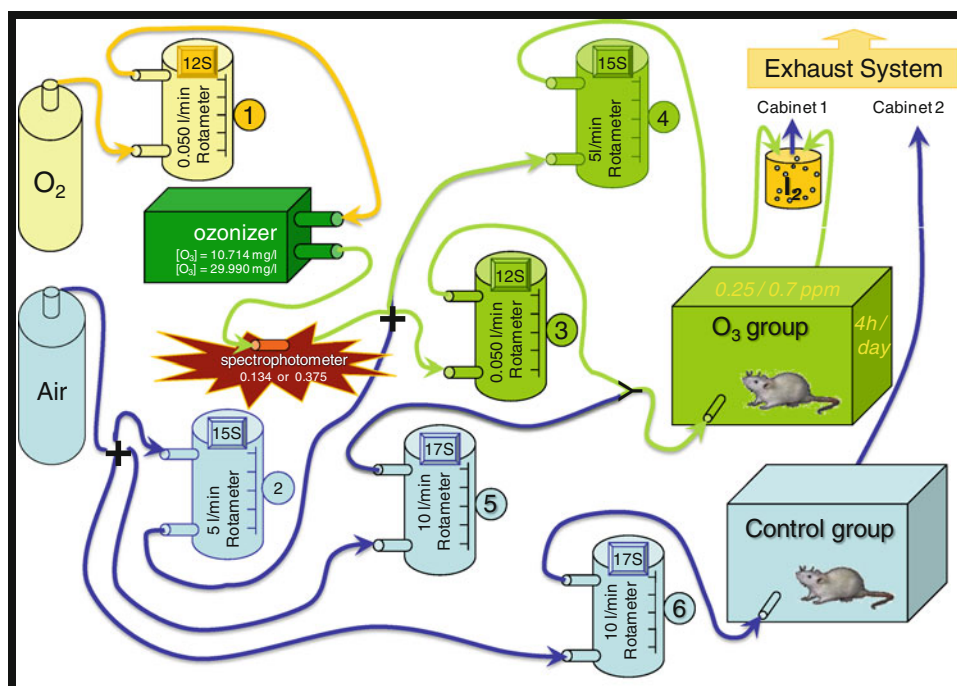
Animals

Male Sprague–Dawley rats (270–310 g) were provided by the Animal Centre of the North-West University (NWU) and the experimental protocol approved in accordance with the regulations set by the Ethical Committee of the North-West University (Ethics number: NWU-00008-07-A8). The rats were housed four rats per cage under controlled conditions of temperature ($22 \pm 1^\circ\text{C}$) and humidity (50%) with a 12:12-h light/dark cycle and food ad libitum.

Experimental design

Scheme 1 represents a graphical outline of the experimental setup for in vivo ozone exposure. A Sander Certizon Ozonizer C100 (100 mg/h capacity; Amtsgericht Hildesheim, Germany) was used to produce ozone from ultra high purity oxygen (99.995%; African Oxygen Limited, Johannesburg, South Africa). The concentration of ozone produced and delivered to the cages was measured and constantly monitored spectrophotometrically, using a Unico 2800[®] spectrophotometer (Dayton, OH, USA) at wavelength 254 nm, path length 2 mm. Under these conditions, and at room temperature (22°C), the ozonizer typically produced ozone at concentrations varying between 10.7 and 30.0 mg/ml. The appropriate ozone

Scheme 1 Experimental design: in vivo ozone exposure (refer to text for detailed description)



concentration (0, 0.25 or 0.7 ppm) for administration to four rats per 60 l Plexi glass chamber was obtained by mixing the ozone in appropriate ratios with compressed medical air (African Oxygen Limited, Johannesburg, South Africa), using appropriately sensitive rotameters from Key Instruments (Trevose, Pennsylvania, USA). A constant flow rate of 10 l/min of the mixed air from the rotameters into the vented chambers allowed for one air exchange every 6 min. All ozonated air (excess from tubing and from the chambers) was bubbled through potassium iodide solution to remove any reactive ozone by forming iodine, and the air discarded through the exhaust system.

Ozone exposure

Acute study

Rats were exposed to 0, 0.25 or 0.7 ppm ozone for 4 h starting at 13:00, initiated directly after the first intraperitoneal injection containing either imipramine (10 mg/kg) or vehicle.

Chronic study, 30 days

Rats received 0 or 0.25 ppm ozone daily for four hours starting at 13:00 (analogous to acute studies), as well as on the penultimate 30th day at the same time, following directly after the first intraperitoneal injection containing either imipramine (10 mg/kg) or vehicle (as in acute studies).

Dissection of the frontal cortex

After the ozone and drug treatments, the rats were decapitated, the whole brain removed and placed in ice-cold double distilled water (ddH₂O), where after the frontal cortex was dissected out on an ice-cooled dissection slab (Chiu *et al.*, 2007) and immediately snap frozen in liquid nitrogen and stored at -70°C until use. On the day the assays were performed, the frontal cortex was thawed, weighed and homogenized in 0.1 M phosphate-buffered saline (PBS) to yield a final tissue concentration of 10% w/v. A method described by Bradford (1976) was used to determine protein quantity (Bradford, 1976).

Superoxide anion assay

Superoxide radical formation was measured according to the standard nitro blue tetrazolium method of Ottino (Ottino and Duncan, 1997) as modified by Harvey (Harvey *et al.*, 2008). In this assay nitro blue diformazan (NBD) is formed in the presence of superoxide radical in the sample.

The absorbance was measured at 560 nm using a spectrophotometer and converted to millimolar diformazan using a standard curve generated from NBD, and the results were expressed as nanomolar/milligram protein.

Lipid peroxidation assay

Lipid peroxidation was measured according to the method described by Ottino and Duncan (1997) and as modified by Harvey *et al.* (2008), but with optimized pH and temperature for our experimental conditions. The malondialdehyde (MDA), a product of membrane lipid peroxidation in response to high ambient levels of ROS, levels were determined from a standard curve generated from 1,1,3,3-tetramethoxypropane. The absorbance was measured at 532 nm using a Spectronic 20 (Bausch and Lomb) spectrophotometer and the results expressed as nmol MDA/mg protein.

Statistical analysis

All the data were analysed first using a two-way analysis of variance (ANOVA) to excluded interaction, where after the one-way ANOVA with the Tukey–Kramer multiple comparison post-test was implemented. GraphPad Prism version 5 was used for data representation and all statistical analysis.

Results

The results obtained from the *in vivo* ozone exposure studies in the rodent are presented in Fig. 4.

Non-specific biomarkers of oxidative stress (superoxide and lipid peroxidation)

Frontal cortex superoxide and lipid peroxidation following acute ozone inhalation (Fig. 4A, B)

Acute imipramine treatment alone did not influence superoxide levels (Fig. 4A, bar a vs. b). Acute exposure to 0.25 ppm ozone, with or without imipramine, failed to alter superoxide levels in the frontal cortex versus control (Fig. 4A, bars c, d vs. bar a). However, acute exposure to 0.7 ppm ozone significantly increased superoxide levels irrespective of the presence or absence of imipramine (Fig. 4A, bars e, f vs. bars a, d; $P < 0.001$). Similarly, acute exposure to 0.25 ppm ozone, with or without acute imipramine treatment failed to alter lipid peroxidation levels in the frontal cortex versus control (Fig. 4B, bars c, d vs. bar a). Acute exposure to the higher dose of 0.7 ppm ozone significantly increased lipid peroxidation levels

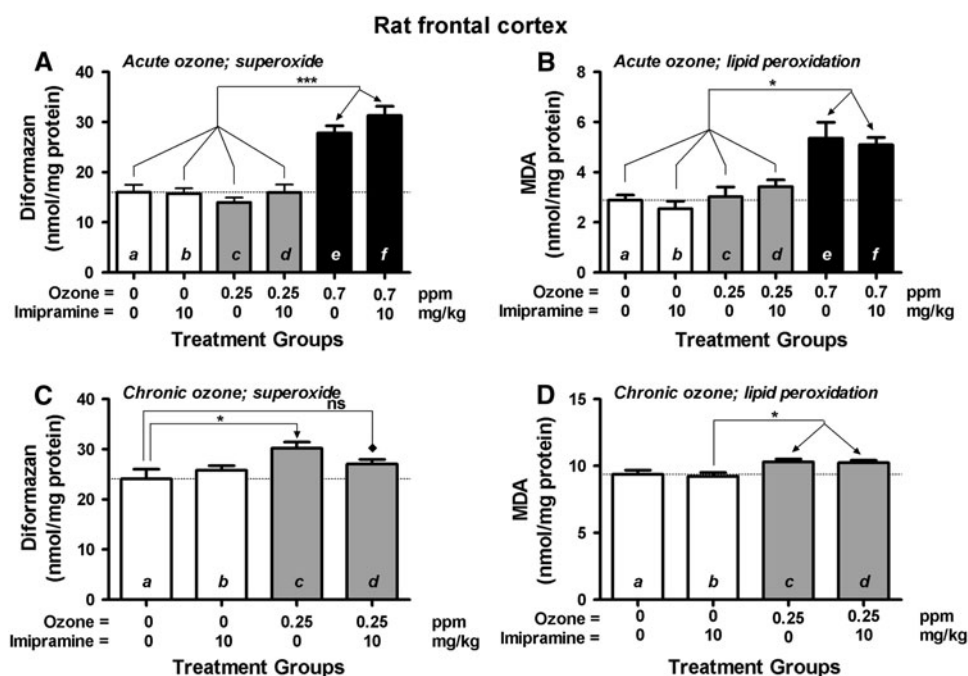


Fig. 4 The effects of acute (4 h) exposure to 0, 0.25 or 0.7 ppm ozone (A, B), or of chronic exposure to 0 or 0.25 ppm ozone (C, D), plus 0 or 10 mg/kg acute imipramine treatment on the indicated parameters in the rat frontal cortex. The indicated parameters were (A, C) levels of superoxide free radical, measuring diformazan (nmol/mg protein) and (B, D) levels of lipid peroxidation, measuring MDA

(nmol/mg protein). Data points represent the mean \pm SE with $n = 7$ for figures A and B and $n = 9$ for figures C and D. Two-way ANOVA analyses indicated no interaction between ozone and imipramine. Therefore, * $P < 0.05$ and *** $P < 0.001$, as determined from a one-way ANOVA, followed by Tukey–Kramer

irrespective of the presence or absence of imipramine (Fig. 4A, bars e, f vs. bars a, d; $P < 0.05$).

Frontal cortex superoxide and lipid peroxidation following chronic ozone inhalation (Fig. 4C, D)

Acute imipramine treatment alone did not influence superoxide levels (Fig. 4C, bar a vs. b). However, similar to acute exposure to 0.7 ppm ozone, chronic exposure to 0.25 ppm ozone significantly increased superoxide levels relative to ozone-free control (Fig. 4C, bar c vs. bar a; $P < 0.005$). Interestingly, however, when acute imipramine treatment is administered after the chronic ozone, the ozone-induced elevation of superoxide levels is reversed, and no longer significantly different from control (Fig. 4C, bar d vs. bar a; not significant). However, chronic exposure to 0.25 ppm ozone elevated lipid peroxidation relative to imipramine alone only (Fig. 4D, bars c, d vs. bar b; $P < 0.05$), but not relative to ozone-free control (Fig. 4D, bars c, d vs. bar a; not significant). In addition, different from superoxide levels, the ozone-induced elevation lipid peroxidation was not reversed by acute imipramine treatment (Fig. 4D, bar d vs. bar a).

Discussion

Biological activity and safety

Numerous reports on the putative medicinal applications of ozone abound in alternative medicine. However, the majority of reports on the clinical application of ozone in humans originate from uncontrolled investigations and as anecdotal evidence. The *in vitro* investigations as well as *in vivo* human reports caution against uncontrolled exposure of airway tissue to ozone. Controlling agencies worldwide have subsequently developed guidelines with ozone exposure limits for humans, in view of the daily ozone exposure measured for the USA reaching levels up to 0.1 ppm (Jerrett *et al.*, 2009). Measurements in the EU showed that some European cities are reaching peak ozone levels of 0.14 ppm (Jerrett *et al.*, 2009). The Environmental Protection Agency (EPA) of the USA set the ozone limit of exposure to an average concentration of more than 0.10 ppm for 8 h (www.epa.gov, US 2010). In South Africa the limits for ozone exposure are set at a maximum of 0.1 ppm for 1 h, or 0.06 ppm for 8 h (South African Bureau of Standards, 2004). It is, therefore, clear that countries are in general concerned that ozone exposure

bears a health risk and in particular to the respiratory system. However, these agencies are not definitive on the possible central nervous system effects of ozone exposure.

Effects of ozone inhalation on non-specific biomarkers of oxidative stress in the brain

Results for this study clearly show that acute and chronic exposure to ozone increases oxidative stress in the rodent frontal cortex, as measured by elevated levels of superoxide and MDA in the frontal cortex. Acute imipramine treatment reverses the levels of superoxide after chronic, but not after acute exposure to ozone. The ozone-induced elevation of oxidative stress in the frontal cortex is associated with concurrently elevated oxidative damage as measured by accumulation of MDA, a product of lipid peroxidation. However, the oxidative damage is more pronounced after acute than after chronic exposure of ozone, since the levels of lipid peroxidation are not significantly elevated relative to ozone-free control after chronic exposure. This may be explained by adaptation and the activation of endogenous antioxidant mechanisms during chronic exposure to ozone and warrants further investigation. Of primary importance for this study, however, is the demonstration of the effects of the ozone inhalation on biological parameters of the cellular redox status in the rat brain and their sensitivity to ozone dose and duration of exposure. Furthermore, it was demonstrated that it is possible to modulate this response by introducing an acute dose of a centrally active psychotropic drug. Our data indicate that acute imipramine treatment can yield some protection against oxidative stress conditions induced by ozone exposure, which has important implications for how imipramine induces its antidepressant effects. Indeed, actions on cellular redox represent a novel target for antidepressant action (Harvey, 2008). The ozone inhalation model can, therefore, be used to study the role of redox pathways in depression and antidepressant action.

Unpublished results from this laboratory (data not shown) indicate that acute exposure to 0.7 ppm ozone or chronic exposure to 0.25 ppm ozone attenuates the antidepressant-like effect of imipramine in the forced-swim test (i.e. animal behavioural data). These data suggest that ozone exposure may in fact attenuate the therapeutic response to an antidepressant drug, thereby negatively influencing the clinical outcome of antidepressant therapy. The ozone inhalation model may be suitable to study the effect of oxidative stress on animal behaviour.

Conclusions

It is clear from the data presented here and from the literature that the ozone is a unique compound with complex

effects on biological systems and other matrixes, including the central nervous system. These investigations not only yielded information on the safety and biological activity of ozone, but also provided novel evidence that ozone possesses unique pharmacological and toxicological properties. In particular, ozone provides an opportunity to investigate the neuropathology of oxidative stress in controlled experimental design, as well as potential novel investigations in drug discovery research. Increasing evidence of the involvement of oxidative stress in several neuropsychiatric and other brain diseases warrants the development of such an *in vivo* model. The ozone inhalation oxidative stress-induced model presented here provides an excellent opportunity to investigate such disorders and drug interventions. These studies also emphasize caution for the systemic use of ozone for medicinal purposes in view of its complex activities and actions not only on peripheral tissue such as the lungs, but also the brain. The potential negative effects of ozone exposure in the presence of other diseases may be associated with greater risk for adverse effects. In addition, we provide evidence that ozone exposure attenuates the beneficial effects of drugs treatments, in this case of antidepressants. It is clear that *in vivo* pre-clinical studies must be significantly expanded to follow up on the numerous *in vitro* studies in order to further drug discovery and to develop ozone as a potential medicinal modality.

Acknowledgments The South African National Research Foundation (NRF) (Grant no. 45846) is thanked for financial support.

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