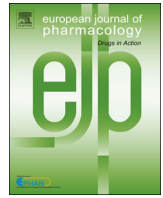




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Is ozone pre-conditioning effect linked to Nrf2/EpRE activation pathway *in vivo*? A preliminary resultLamberto Re^{a,*}, Gregorio Martínez-Sánchez^b, Marica Bordicchia^c, Giuseppe Malcangi^a, Antonella Pocognoli^c, Miguel Angel Morales-Segura^d, John Rothchild^e, Armando Rojas^f^a Clinical Pharmacology & Toxicology Department, Medinat SAS, Via Fazioli 22, 60021 Camerano, Ancona, Italy^b Medical Center Beauty Benefit – San Biagio di Osimo, Via Mons. Oscar Romero, 31, 60027 Osimo, Ancona, Italy^c Department of Internal Medicine, University of Ancona, Politecnica delle Marche, 60131 Ancona, Italy^d Department of Pharmacology, Faculty of Medicine, University of Chile, Santiago de Chile, Chile^e Holistic Dentist Clinic, 175 Mercado Street, Suite 115, Durango, CO 81301, USA^f Biomedical Research Laboratories, Medicine Faculty, Catholic University of Maule, Talca, Chile

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ABSTRACT

The present preliminary study has been focused on verifying whether ozone preconditioning may be linked to Nrf2/EpRE (nuclear factor erythroid 2/electrophile-responsive element) activation pathway *in vivo*. Healthy volunteers received a total of three Major Auto-Hemotherapy (MAH) treatments, with treatments administered every second day. The amount of blood used for each subject was standardized to the value obtained multiplying the subject's body weight by 1.3 in order to ensure the same ozone concentrations for each subject. A parallel group ($n=50$) age and gender matched was used as reference for the experimental variables related to the oxidative stress parameters. Levels of Nrf2 and oxidative stress index were measured throughout the study. Levels of Nrf2 ($P < 0.01$) in peripheral blood mononuclear cells (PBMC) were found to increase immediately after ozone/oxygen exposure (35 $\mu\text{g}/\text{ml}$, prior to reinfusion). This effect was still detected ($P < 0.05$) in total circulating PBMC when measured 30 min following reinfusion. After a series of 3 MAH, Nrf2 returned back to the basal level. At the end of the experiment the activities of superoxide dismutase and catalase were increased ($P < 0.05$). These data demonstrate for the first time *in vivo* the activation of the Nrf2 pathway by a low dose of ozone and the promotion of the feedback mechanism that induces the synthesis of proteins which collectively favors cell survival.

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1. Introduction

Despite long experience in the field of medical ozone therapy the putative molecular mechanisms underlying some reported clinical effects of this gas remain mainly unknown. Nevertheless, further research is still lacking and the most intimate mechanisms underlying ozone's effects remain partially ignored.

The present study has been focused on verifying whether ozone preconditioning may be linked to Nrf2/EpRE (nuclear factor erythroid 2/electrophile-responsive element) activation pathway. It is tempting to speculate that this system could be activated by a controlled ozone-induced oxidative stress, which may partially explain the effects reported for ozone therapy.

The Nuclear factor, Nrf2 is a master transcription factor that regulates EpRE, an antioxidant response element (ARE) or human ARE (hARE) in human, mediated expression genes encoding antioxidant enzymes and cytoprotective proteins to cellular stresses (Brigelius-Flohe and Flohe, 2011). These Nrf2-regulated genes can be classified into phase II xenobiotic-metabolizing enzymes antioxidants, molecular chaperones, DNA repair enzymes, and anti-inflammatory response proteins, thereby reducing reactive compounds such as free radicals to less toxic intermediates helping the cell in repairing any ensuing damage. Importantly, Nrf2 has been shown to possess an EpRE sequence within its own promoter region providing a platform for Nrf2 to initiate its own transcription further enhancing the adaptive cell defense response (Brigelius-Flohe and Flohe, 2011).

Recent studies suggest that the sequence context of the EpRE, the nature of the chemical inducers, and the cell type are important for determining the activity of the enhancer in a particular gene (Pecorelli et al., 2013). Evidence of the EpRE pathway was observed

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also for some xenobiotics modulating the regulation of Phase I and Phase II drug-metabolizing enzymes (Prochaska et al., 1985).

Nrf2 is a powerful protein located within each cell in the body and it is activated by an Nrf2 activator. Once released it migrates into the cell nucleus and bonds to the DNA at the location of the EpRE which is the master regulator of the entire antioxidant system located in all human cells (Kobayashi et al., 2009). Nrf2 is located in the cell's nucleus and could be activated by many sources of oxidative stress including free radicals. The same factor then induces a cascade of events activating the most important antioxidants (de Vries et al., 2008). We know that excessive free radicals induce an antioxidant response through this pathway, but the question is whether the molecular mechanism of ozone *in vivo* is connected with Nrf2.

Previous studies have demonstrated that such a response does occur *ex vivo* (Pecorelli et al., 2013). The purpose of this study is to determine whether a similar response occurs in the patient's blood *in vivo*. Is ozone able to induce and trigger throughout Nrf2 the production of thousands of antioxidant molecules, and thus inhibit the damaging effects of free radicals? The aim of the present study was to assay the levels of Nrf2 in peripheral blood mononuclear cells (PBMC) and redox index in healthy subjects after a small dose of ozone to demonstrate *in vivo* the involvement of the Nrf2/EpRE pathway.

2. Materials and methods

The study was a mono-centric, prospective and controlled clinical trial approved by the institutional review board (Scientific and Bioethics Committees of the Institution, Medinat Clinic) in accordance with the Declaration of Helsinki (WMA, 2004). All patients gave their informed consent to being enrolled after receiving adequate information about the study (characteristics of the study, benefits and possible side effects). Before enrolling, all participants attended a training program to familiarize them with the study objectives and treatment plans. The personnel involved emphasized that all participating physicians would treat each patient according to the scheme of treatment.

Six adult healthy Caucasian subjects of both genders (3 males and 3 females) were eligible to participate in the study. The volunteers enrolled for the study underwent the designed protocol at the Medinat Clinic (Ancona, Italy) from June 19th to July 3rd 2013. Exclusion criteria were previous treatment with ozone therapy, hypersensitivity to the medication to be used, hepatic dysfunction, renal failure (serum creatinine level > 1.32 $\mu\text{mol/l}$), pregnancy, cancer or other acute or chronic serious diseases, inability to cooperate with the requirements of the study, recent history of alcohol or drug abuse, current therapy with any immune-suppressive agent or anticonvulsant, concurrent participation in another clinical study, or current treatment with an investigational drug or any drug linked to the induction of oxidative stress.

Major Auto-Hemotherapy (MAH) was performed with Ozonosan[®] Bottles and ozone. Ozone was generated from medical-grade oxygen (O₂) using electrical corona arc discharge, by the O₃ generator (Model Ozonosan Alpha Plus, Hansler, GmbH, Iffezheim, Germany), which allows the gas flow rate and O₃ concentration to be controlled in real time by photometric determination, as

recommended by the Standardization Committee of the International O₃ Association. Briefly, blood was drawn from the subjects, exposed to the same volume of oxygen or ozone and re-injected into the subjects. The amount of blood used for each subject was standardized to 1.3 multiplied by the body weight (BW) of each patient in order to have the same ozone concentrations per kg BW. Patients were treated with 3 MAH, one every 2 days interval.

Data from 50 gender- and age-matched healthy subjects not involved in this study were used as reference to establish the normal (control) range interval of the experimental variables (oxidative stress bio-markers). Control values were obtained using the same methods employed to analyze samples of subjects involved in this study.

Blood Sampling to assay Nrf2 and oxidative stress variables is shown in Fig. 1.

2.1. Determination of Nrf2-antioxidant response element binding efficiency

For each patient mononuclear cells were separated from whole blood using Histopaque[®]-1077. Then viable cells, checked by MTT assay (Mosmann, 1983), were immediately processed to obtain nuclear protein extract using Nuclear Extract kit (Active Motif, Carlsbad, CA). Immediately, nuclear extract was aliquoted and stored at $-80\text{ }^{\circ}\text{C}$ until analysis with Trans-AM Nrf2 kit (Active Motif, Carlsbad, CA). Briefly, 10 μg of nuclear protein was incubated in 96-well plates pre-coated with ARE consensus oligonucleotides (5'-gtcacagtactcagcagaatctg-3'), and the active-Nrf2 that bounds to the oligonucleotides was detected by incubating with anti-Nrf2-antibody and appropriate HRP-conjugated secondary antibody. Absorbances were read at 450 nm using a plate reader (Victor, Perkin-Elmer), the detection limit of the kit was < 0.15 μg nuclear extract/well and the range of detection: 0.15–2.5 μg nuclear extract/well. Absorbance was expressed as the direct activity of Nrf2.

2.2. Biochemical assays

Blood samples were immediately centrifuged at 2000g, at 4 $^{\circ}\text{C}$ for 15 min and plasma was stored at $-80\text{ }^{\circ}\text{C}$ until biochemical analysis. All biochemical parameters were determined by spectrophotometric methods using an UviLine 9400 Spectrophotometer (Secoman, France). Catalase activity was measured by following the decomposition of hydrogen peroxide at 240 nm at 10 s intervals for 1 min (Haining and Legan, 1972). Superoxide dismutase was measured using kits supplied by Randox Laboratories Ltd., Ireland (Cat. nos. SD125 and RS505). Concentrations of malondialdehyde were analyzed using the LPO-586 kit obtained from Calbiochem (La Jolla, CA). In the assay, the production of a stable chromophore, after 40 min of incubation at 45 $^{\circ}\text{C}$, was measured at 586 nm. For standards, freshly prepared solutions of malondialdehyde bis [dimethyl acetal] (Sigma, St. Louis, MO, USA) were used and assayed under identical conditions (Esterbauer and Cheeseman, 1990). Quantification of total hydroperoxides was measured by Bioxytech H₂O₂ – 560 kit (Oxis International Inc., Portland, OR, USA) using xylene orange to form a stable colored complex, which can be measured at 560 nm.

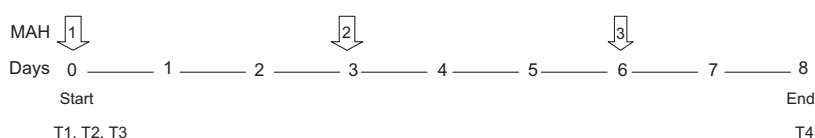


Fig. 1. The graphical time schedule of the ozone treatment.

After precipitation of thiol proteins using trichloroacetic acid 10%, reduced glutathione was measured according to the method Sedlak and Lindsay (1968) with Ellman's reagent [5,5 dithiobis (2-nitrobenzoic acid) 10^{-2} M (Sigma, St. Louis, MO, USA)]; absorbance was measured at 412 nm. The advanced oxidation protein products (AOPP) were measured as the oxidation of iodide anion to diatomic iodine by advanced oxidation protein products (Witko-Sarsat et al., 1998). Ferric reducing ability of plasma (FRAP) was assayed through the reduction of Fe^{3+} to Fe^{2+} by serum or reference (ascorbic acid). The Fe^{2+} -2,4,6-tripiridil-s-triazine complex was detected at 593 nm (Benzie and Strain, 1996).

2.3. Statistical analysis

The OUTLIERS preliminary test for detection of error values was initially applied. Afterward, biochemical data were analyzed by one-way analysis of variance (ANOVA) followed by a homogeneity variance test (Bartlett-Box). In addition, a multiple comparison test was used (Duncan test).

Results are presented as mean \pm standard deviation. Values of Nrf2 were analyzed using 2-tailed Student's *t* test and presented as mean \pm SEM unless otherwise indicated. The level of statistical significance used was $P < 0.05$. Prism 5.0 software was used for statistical analyses; *P* values for significance are indicated for each data set.

3. Results

The effect of ozone on Nrf2 activation in PBMC is shown in Fig. 2. Total blood exposed to a mixture of ozone (35 $\mu\text{g}/\text{ml}$) and oxygen increased Nrf2 ($P < 0.01$) in PBMC immediately after the MAH. This effect was maintained when measured 30 min following reinfusion, when the increment in Nrf2 was detected ($P < 0.05$) in total circulating PBMC. After a series of 3 MAH (every 2 days interval), Nrf2 returned back to the basal level. No gender differences were found between the values of Nrf2 in the time intervals assayed.

Basal data of the study group shows the presence of a low oxidative stress characterized by low levels of GSH and total antioxidant activities (FRAP) and high levels of AOPP ($P < 0.05$) compared to reference group. However the activities of antioxidant enzymes (CAT and SOD) were found in the normal ranges compared to reference group.

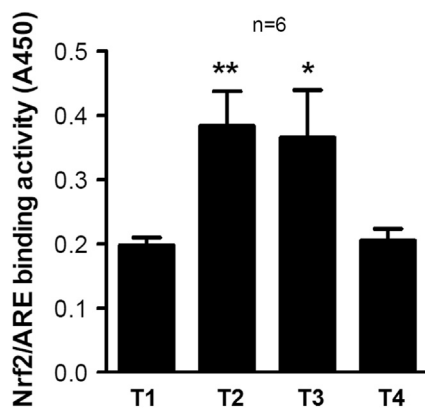


Fig. 2. Peripheral blood mononuclear cells transcription factor binding (Trans-AM-Nrf2 activity) before and after blood ozonization. T1, immediately before the first Major Auto-Hemotherapy (MAH); T2, from the bottle during the first MAH after bubbling blood with ozone; T3, from the patients 30 min after the first MAH; T4, from the patients one day after the application of the third MAH. Data are mean \pm S.E.M. * $P < 0.05$ ** $P < 0.01$ vs. T1.

Index of bio-molecules oxidation (MDA, AOPP) was increased ($P < 0.05$) immediately after MAH (Table 1) and returned back to the basal level after the cycle of MAH. No changes were detected after MAH in the levels of the total hydroperoxides. Concentration of GSH was depleted ($P < 0.05$) immediately after the reaction of ozone with blood (T2) but levels in the total blood after the re-infusion (T3) get similar values compared to basal levels (T1). The same shape was observed for the index of total antioxidant activity FRAP. Antioxidant enzymatic system (catalase and SOD) increases its activity ($P < 0.05$) after the cycle of 3 MAH.

4. Discussion

Evidence of the involvement of Nrf2 in the molecular mechanism of ozone can be found in previous toxicological and pharmacological assays (Kim et al., 2004; Cho et al., 2013). Ozone induced an increment of Nrf2 (Kim et al., 2004) in lungs and livers of B6C3F1 mice. The role of Nrf2 in airway toxicity caused by inhaled O_3 in mice was studied in Nrf2-deficient (Nrf2(-/-)) and wild-type (Nrf2(+/+)) mice received acute and subacute exposures to O_3 . Results suggest that Nrf2 deficiency exacerbates oxidative stress and airway injury caused by the environmental pollutant O_3 (Cho et al., 2013). In addition, the *in vivo* study of the effect of ozonized saline (preconditioning schedule) on the activation of the Keap1-Nrf2-EpRE signaling pathway demonstrated the reduction of rat's liver injury induced by CCl_4 . The ozonized saline, as a novel Nrf2 activator, can reduce the oxidative damage of radical oxygen species (ROS) and the deleterious substances by activating the Keap1-Nrf2-EpRE signaling pathway and its downstream genes expression (Qu et al., 2011). Meanwhile, oxygen in hyperoxia during the newborn period can induce lung injury by a mechanism mediated by Nrf2 (Cho et al., 2012).

The results on ozonized serum (an *ex vivo* experiment) demonstrate a dose related activation of Nrf2 and the subsequent induction of HO-1 and NADPH quinone oxidoreductase in endothelial cells culture (Pecorelli et al., 2013). Currently, scientific evidence links the modulation of different biomarkers (e.g. antioxidant enzymes, nitric oxide pathways and 2,3-diphosphoglycerate) as a consequence of applying low ozone doses (Re et al., 2012; Viebahn-Hänsler et al., 2012). Those facts support some of the current clinical applications of ozone therapy as an integrative supplement (Re et al., 2008). Ozone can increase the level of nuclear translocated Nrf2, which is associated with an increase in Nrf2 protein translocation from the cytoplasm to the nucleus. As a consequence Nrf2 increased the activity of the antioxidant and phase II detoxifying enzymes (Table 1). The effect can be dissociated from the effect of oxygen, because control sample (T1) values were obtained after the treatment of blood with oxygen (vehicle of ozone).

Furthermore, a recent study well describes the role of Nrf2 as the transcription factor (TF) involved in one of the major adaptive stress response pathways to xenobiotics (Simmons et al., 2009) as that of the oxidative stress.

A reference group is essential to handle experimental variables and establish a comparison with experimental groups. In the subjects under study at T1, the low levels of GSH and FRAP and high levels of AOPP compared to reference group may show that the subjects under study were exposed to a low oxidative stress even when they were in an apparently healthy state.

MAH is one of the most used techniques for the administration of ozone and it involves the exposition of human blood (100–150 ml) to a mixture of O_2 - O_3 , in a range of ozone concentration of 0.21–1.68 μM per ml blood (Pecorelli et al., 2013). In the present work the dose of ozone was 0.735 $\mu\text{M}/\text{ml}$. Ozone in contact with blood instantaneously (2 min approximately) reacts with antioxidant and bio-molecules (lipid and proteins) generating different

Table 1
Biomarkers of antioxidant/pro-oxidant balance.

Biomarkers	Control values ^a	T1	T2	T3	T4
AOPP (μM of chloramine)	10.38 ± 1.77 ^a	14.93 ± 4.84 ^b	23.15 ± 8.26 ^c	23.44 ± 2.35 ^c	15.27 ± 3.67 ^b
MDA (μM)	1.80 ± 0.07 ^a	1.73 ± 0.19 ^a	2.11 ± 0.35 ^b	1.82 ± 0.35 ^a	1.75 ± 0.17 ^a
Total hydroperoxide (μM)	103.78 ± 17.71 ^a	104.02 ± 27.11 ^a	94.96 ± 18.08 ^a	89.58 ± 17.44 ^a	97.80 ± 22.93 ^a
GSH (mM)	2.56 ± 0.31 ^a	1.27 ± 0.94 ^b	00.78 ± 0.47 ^c	1.09 ± 0.29 ^b	1.05 ± 0.29 ^b
FRAP (μM)	1017.1 ± 206.0 ^a	268.12 ± 38.87 ^b	188.89 ± 22.28 ^c	256.98 ± 39.68 ^a	261.55 ± 37.31 ^a
Enzymes	Control Values^a	T1		T4	
SOD (U/mL/min)	1.46 ± 0.14 ^a	1.50 ± 0.90 ^a		3.14 ± 1.07 ^b	
CAT (U/L/min)	161.5 ± 23.11 ^a	195.00 ± 62.75 ^a		560.63 ± 44.73 ^b	
CAT /SOD	0.11 ± 0.20 ^a	00.13 ± 0.06 ^a		00.18 ± 0.04 ^b	

AOPP, advanced oxidation protein products; GSH, reduced glutathione; MDA, malondialdehyde; PP, peroxidation potential; SOD, superoxide dismutase; CAT, catalase; FRAP, ferric reducing ability of plasma; sampling time: T1, from the patients immediately before the first MAH; T2, from the bottle during the first MAH after bubbling blood with ozone; T3, from the patients 30 min after the first MAH; T4, from the patients the day after the application of the third MAH. Data are mean ± S.D. Means having different superscript letters indicate significant difference ($P < 0.05$) comparing reference, initial and final times of each group value between the same set.

^a Laboratory data for healthy individuals ($n=50$). This group of subjects corresponded in terms of age, gender and ethnicity with patients enrolled in the study.

bio-products, mainly H_2O_2 and aldehydes (Pecorelli et al., 2013). As shown in Table 1, levels of AOPP and MDA were increased ($P < 0.05$) after blood ozonization; however antioxidant (GSH) and the index of total antioxidant FRAP decreased ($P < 0.05$). Moreover, no changes in total hydroperoxides levels were detected, probably due to the short half-life of this kind of compound (around 20 s) (Pecorelli et al., 2013).

Two days after the 3rd MAH (time T4) the recovery of the patients from the initial low oxidative stress is not reached. Despite clinically suggested protocols which are based upon a series of 10–15 applications we did the measurement after only 3 MAH for experimental purposes. However, a significant increment of SOD and CAT was observed also under these experimental conditions. The assayed sample is too short to launch a hypothesis about this behavior.

The effect induced by ozone preconditioning (Leon et al., 1998) has been described by several authors (Re, 2008; Bocci et al., 2011) as hormesis phenomena (Calabrese et al., 2011). The hormesis concept is well described by a U shaped relationship in which effects at low doses are opposite to those at high doses. As a consequence, hormetic dose response curves are biphasic rather than being monotonic and our effort in the field of ozone therapy is to look for the optimal dose and not for dose-related effects (Calabrese et al., 2011). This was the reason for which we employed a single ozone conditioning concentration.

Kelch-like ECH-associated protein 1 (Keap1) plays a pivotal role in responding to oxidative challenge with adaptive response via activation of the transcription Nrf2 (Brigelius-Flohe and Flohe, 2011). Ozone in contact with blood components (serum component and cells membrane) generates oxidative bio-products that, entering in the cell cytosol, can oxidize cysteines residues in Keap1. This fact has been demonstrated for 4-hydroxynonenal (Ishii et al., 2004) and some other aldehydes (Levonen et al., 2004). Oxidation of Zn-coordinated cysteines leads to release of Zn and consecutive unfolding of the Keap1 finger structure. The structural change of Keap1 then prevents ubiquitination and proteosomal degradation (Brigelius-Flohe and Flohe, 2011). Nrf2, via activation of EpRE, regulates the expression of proteins that collectively favor cell survival.

Taking into account all data presented herein, we can suggest ozone as a useful tool to integrate and complement (Re et al., 2012) the pharmacological therapy modulating the oxidative stress component in many illnesses and considered as a strategic allied of the orthodox pharmacological treatment.

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