Journal of Applied Pharmaceutical Science Vol. 3 (11), pp. 035-039, November, 2013
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2013.31107
ISSN 2231-3354

Evaluation of intraperitoneal ozone application effects to rat's antioxidant enzymes, superoxide dismutase and glutathione peroxidase

Yannis V Simos[1*], Ioannis K Toliopoulos[1,2], Ioannis I Verginadis[1], Ilias V Karagounis[1], Pavlos S Charalampidis[1,3], Vasilios N Ragos[4], Angelos M Evangelou[1], Spiros C Karkabounas[1]

[1]Laboratory of Physiology, Medical School, University of Ioannina, Ioannina, Greece. [2]Konstantinion Research Center of Molecular Medicine and Biotechnology, non-profit foundation, Thessaloniki, Greece. [3]Strimonikos Health Center, Serres, Greece. [4]Maxillofacial Surgery, Faculty of Medicine, University of Ioannina, 45110 Ioannina, Greece.

ARTICLE INFO

Article history:
Received on: 13/09/2013
Revised on: 14/10/2013
Accepted on: 06/11/2013
Available online: 29/11/2013

Key words:
Ozone, oxidative stress, antioxidant enzymes, total antioxidant capacity.

ABSTRACT

Ozone is a powerful oxidant that presents dual activity: either as a therapeutic or as a toxic agent. The aim of this study was to investigate the effects of intraperitoneal (i.p.) application of ozone on the endogenous antioxidant enzymes of Wistar rats compared with the amendment of the total antioxidant capacity in blood and urine. Rats were divided randomly into two groups: the control group (CG) which was injected with placebo (NaCl 0.9%) and the ozone group (OG) which was administrated daily for 10 consecutive days with ozone at a concentration of 6.3μg/Kg body weight. Blood and urine samples were collected at the beginning, after the administration period and 22 days after the initiation of the experiment. Intraperitoneal application of ozone leads to a potent Increase to glutathione peroxidase (GPx) levels with no effect to Superoxide dismutase (SOD) levels. Total antioxidant capacity (TAC) was significantly decreased in plasma, red blood cells (RBCs) and urine of the rats. Malondialdehyde (MDA) concentration in urine remained stable. To our knowledge these are the first experimental results showing that repeated i.p. application of ozone in low doses produce severe changes to the whole antioxidant capacity of the blood and indications of toxicity, as the organism exert efforts to adapt to oxidative stress.

INTRODUCTION

Hormesis is a phenomenon where low exposure to a stressor agent produces favourable biological responses (Calabrese, 2011). Hormesis has been observed literally across the biological spectrum, including medicine, nutrition, pharmacology, toxicology, physiology etc (Calabrese, 2008; Lindsay, 2005; Zhang *et al*. 2009; Shanker, 2008; Radak *et al.* 2008). Ozone is one of the most potent oxidants and prolonged inhalation can be harmful for the organism. It has also been proposed that small doses of ozone can trigger several biochemical mechanisms; it is claimed that through ozone therapy, the ozonated blood produces "messengers" which are transferred to cells producing a therapeutic effect (Bocci, 2007). This concept is based on an aspect of the antioxidant network operation. Specifically, part of the antioxidant system is characterised by its ability to adapt to various signals (Noguchi *et al.* 2008). These signals, responsible for the production and the reactions of free radicals, cause the formation and transfer of appropriate antioxidants to the correct location (Stark, 2008). If the stress (produced by the signal) is tolerable, the cell or the organism can adapt and survive.

Ozone due to its high solubility, rapidly dissolves into plasma or into the extracellular fluids and reacts immediately with a number of molecules present in biological fluids (antioxidants, proteins carbohydrates and polyunsaturated fatty acids-PUFAs) generating primarily reactive oxygen species (ROS) and secondarily lipid oxidation products (LOPs) (Bocci, 2005a). Thus, exposure of an organism to low doses of ozone leads to the production of LOPs that act as long-distance messengers, transmitting to all the organs the information of acute oxidative stress (Bocci, 2002). The most common recommended applications of ozone include: a. systemic: major autohemotherapy, rectal insufflation and minor autohematotherapy and b. topical: ozone

* *Corresponding Author*
Yannis V. Simos, Clinical Dietician-Nutritionist, MSc (MedSci), PhD, Laboratory of Physiology, Faculty of Medicine, University of Ioannina, Greece, 45110, Tel: +30-26510-07602, Fax: +30-26510-07850,

bagging, the sunction cup method, rectal/vaginal insufflation, intraarticular injection, ozonated water sprays and ozonated ointments (Altman, 2007). Intraperitoneal application of ozone is considered an experimental but very promising method (Altman, 2007). In this context, we followed an *in vivo* approach to evaluate ozone effects which was applied intraperitoneally at extremely low doses. We then analysed ozone effects on the RBCs's antioxidant enzymes, superoxide dismutase and glutathione peroxidase, activity in conjunction with the effects on the total antioxidant activity.

MATERIALS AND METHODS

Chemicals and Reagents

Superoxide dismutase assay kit, Glutathione Peroxidase assay kit, 2-thiobarbituric acid (TBA), 3-methylbutanol, ammonium dichromate, α-tocopherol and hydrogen peroxide of high purity were purchased from Sigma–Aldrich (St. Louis, MO, USA). 1,1,3,3-tetraethoxypropane, phosphoric acid, 3-methylbutanol and hydrogen peroxide of high purity were purchased from Fluka (St. Louis, MO, USA). Disposable polysterene square cuvettes of 10mm optical pathway were from Sarsterdt Co.

Equipment

Equipment instrument systems used for ozone production was Medozons BM, Arzamas Instrument Plant (ozone:oxygen mixture 95-5%). SOD and GPx assay kit as well as TAC measurements were performed on a UV/Vis microplate and cuvette spectrophotometer (Multiskan Spectrum, Thermo Scientific). Analysis of MDA was performed by Liquid Chromatography-Mass Spectrophotometry (LC-MS) (Shimadzu LC-MS 2010EV). Equipment used for blood analysis was the Biocode Hycel Celly 70.

Animals care

Twenty four female Wistar rats 14 weeks' old (3½ months) were used in this study. Their average body weight was 189gr±14gr. Animals were kept in metabolic cages, one animal per cage, in room temperature, under 12/12 h of light/darkness conditions. Standard rat chow (Viozois S.A., Animal Feed Company of Epirus, Greece) and water *ad libitum* were used in all the experiments.

Experiments on animals were handled with human care in accordance with the National Institutes of Health guidelines and the European Union directive for the care and the use of laboratory animals (Greek presidential decree No. 160 1991) and according to the permission number 20EEP02.

Study protocol

The animals were divided randomly into 2 groups (12 animals per group) and received treatment for 10 consecutive days. Rats were left for acclimatization in the metabolic cages for 5 days before Day 0. Animals in the Control Group (CG) were given a single dose of 1.2 cm^3 saline for the experimental period (Day 1-Day 10), whereas animals in Ozone Group (OG) were given 1.2 cm^3 of ozone (1.2μg) (Day 1-Day 10). Food consumption, water intake, urine output and fecal weight were measured daily. Rats were weighted and urine and blood samples were collected at Day 0, 11 and 22. Samples were stored at -80^oC until used. Additionally, metabolic cage temperature, environmental temperature, humidity and water evaporation were recorded daily. All treatments were given in a single intra-peritoneal dose between 11.00 to 12.00 hours.

Treatment period lasted 10 days (Day 1-10) and follow up another 10 days (Day 12-22). Total experimental time was set at 22 days.

Blood samples collection and processing

Blood samples (2ml each) were collected in heparinized tubes (Day 0, 11 and 22) from the jugular vein of the rats. Samples were analyzed immediately for red blood cell (RBC) count, hemoglobin (Hb) and hematocrit (Htc). The samples were also centrifuged at 800 x g for 30minutes in order to separate plasma and red blood cells. Plasma and RBCs samples were stored at -80 ^oC until used.

Assay of Total Antioxidant Capacity

For the evaluation of total antioxidant capacity in plasma, RBC and urine the Blue CrO_5 assay was used according to Charalampidis *et al.* 2009.

Assay of erythrocyte superoxide dismutase and glutathione peroxidase activity

Superoxide dismutase and GPx activity were determined spectrophotometrically using the Sigma-Biochemica 19160 kit, and Glutathione Peroxidase Cellular Activity Assay kit, CGP1 respectively (Sigma-Aldrich Ltd, St. Louis, USA).

Assay of Lipid Peroxidation

Malondialdehyde levels in urine were determined by LC-MS of the adduct obtained with thiobarbituric acid reagent, based on the methodology proposed by Hong *et al.* 2002.

Statistical analysis

Data distribution analysis was performed with the Shapiro–Wilk test. The analysis showed that there was a normal distribution between data.

The statistical significance difference, between data means, was determined by Student's t-test and one-way analysis of variance (ANOVA) was used for statistical evaluation of differences between groups, at different time intervals (SPSS version 17.0, Statistical Package for the Social Sciences software, SPSS, Chicago, USA). p-values $p<0.05$ were considered as significant.

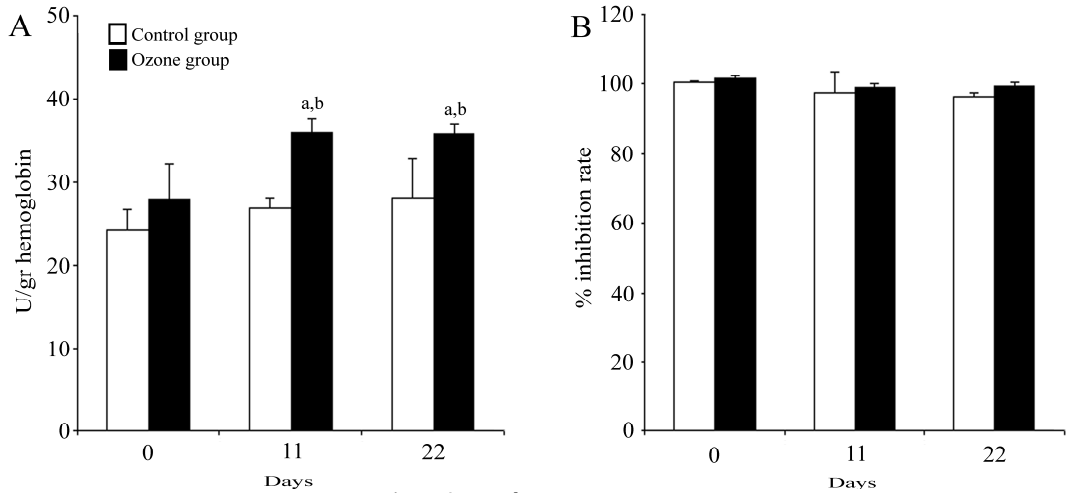

Fig. 1: Antioxidant enzymes activity.
(A) Glutathione Peroxidase activity. (B) Superoxide dismutase activity. Enzyme activity for GPx is expressed as U/gr hemoglobin and for SOD as % inhibition rate. [a]Significantly different from Day 0, $p<0.05$. [b]Significantly different between groups, $p<0.05$. Data are expressed as mean±S.D.

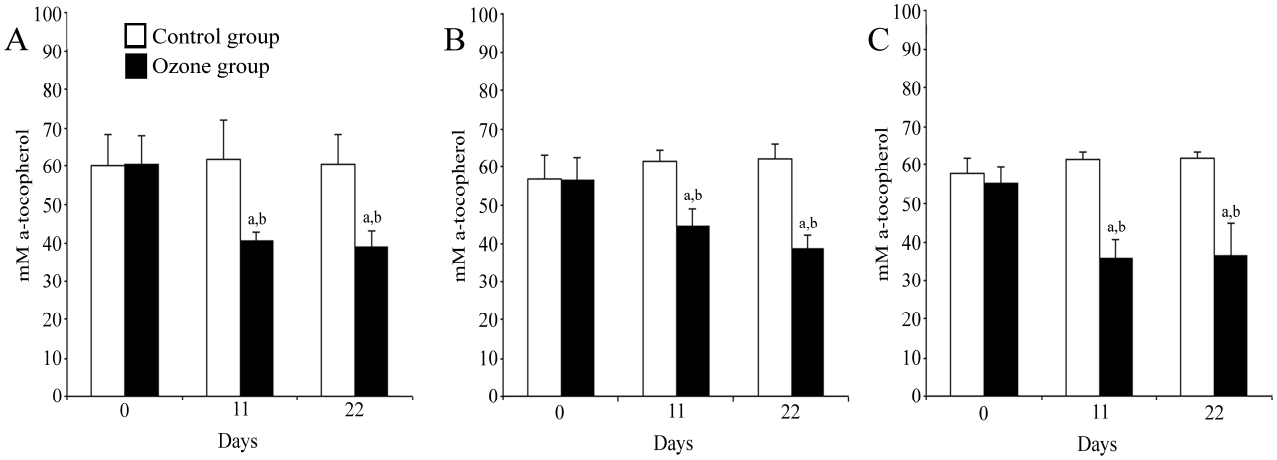

Fig. 2: Total antioxidant capacity.
(A) Total antioxidant capacity in plasma. (B) Total antioxidant capacity in RBCs. (C) Total antioxidant capacity in urines. [a]Significantly different from Day 0, $p<0.05$. [b]Significantly different between groups, $p<0.05$. Data are expressed as mean±S.D.

RESULTS

Ozone administration led to a steep increase in glutathione peroxidase activity (Fig. 1A). After the administration period glutathione peroxidase activity was significantly increased by 29% ($p<0.05$), compared to D0, and remained significantly elevated until the end of the monitoring period (28%) ($p<0.05$). On the other hand, SOD levels remained stable throughout the experiment (Fig. 1B). No differences in the activity of the two enzymes were recorded in the control group (Fig. 1).

Ozone significantly reduced TAC in rats' plasma, red blood cells and urines (Fig. 2). Specifically, after the administration period TAC was significantly reduced by 33%, 21% and 35% in rats' plasma, red blood cells and urine, respectively ($p<0.05$). Rats were incapable to restore TAC levels and this was recorded at the end of the follow up period where levels remained significantly depressed (35%, 31% and 34% in rats' plasma, red blood cells and urines, respectively) ($p<0.05$). As it was expected, TAC levels remained stable in the control group. Moreover, although TAC was significantly reduced in urine MDA levels were not affected (Table 1).

Table. 1: Urine MDA levels in the Control and the Ozone group.

MDA (μM)	Day 0	Day 11	Day 22
Control Group (n=12)	1.62±0.16	1.46±0.22	1.44±0.22
Ozone Group (n=12)	1.91±0.39	1.84±0.24	1.79±0.39

Data are presented as mean ± SD

DISCUSSION

The aim of this study was to investigate the *in vivo* effects of low ozone i.p. administration to the enzymatic activity of the SOD and GPx in relation with the alterations to the TAC of blood.

The therapeutic doses of ozone at ozonated autohemotherapy have been defined between 10 and 80 μg/ml (0.21-0.68 μmol/ml) ozone per ml of blood (Sagai and Bocci,

2011). This "therapeutic window" ensures the medical efficacy of ozone, avoiding toxicity. It has been proposed that low doses of ozone signal the production and the reactions of free radicals which in turns cause the formation and transfer of appropriate antioxidants in the correct location (Bocci, 2005b). Thus, by this way ozone actually acts as a pro-drug; it produces the messenger H_2O_2 and eventually LOPs and rapidly disappears. According to Bocci *et al*., when the ozone dose is well within the defined range (10 and 80 μg/ml) the organism redox system can rapidly restore the decrease of the antioxidant capacity of plasma (Bocci, 2007). An acceptable decrease is no more than 25% and the time required for the reconstitution of the antioxidant capacity of plasma no more than 20 minutes.

From our data, i.p. ozone application at a very low dose (1.2μg/once a day for 10 days) had a significant impact on blood antioxidant capacity. The peritoneum is a cavity with an extensive network of blood vessels. Ozone is dissolved rapidly in the blood stream and the surrounding tissues, ozonating possible, the maximum amount of blood. This resulted in a stiff drop in the antioxidant capacity not only of plasma (more than 25%), but also of RBCs, and remained depressed even 10 days after the end of the experimental period. Even if we arbitrary assume that 1.2μg of ozone were dissolved in 11-13ml of the rat's blood (Lee and Blaufox, 1985) this resulted in an ozone concentration of 0.11-0.09μg/ml, respectively (6.3μg/kg). Kesik *et al*., administered ozone at a dose of 0.72mg/kg daily via i.p. route for 15 days to study the efficacy of ozone therapy in the prevention of methotrexate-induced intestinal injury in rats (Kesik *et al.* 2009). Although methotrexate leads to a potent decrease in SOD and GPx activity and an increase in MDA levels in the intestinal tissue, pretreatment with ozone alleviated methotrexate effects by boosting SOD and GPx activity and by maintaining MDA levels stable. The same results are also observed in the liver and kidney samples, and the authors concluded that ozone preserved antioxidant enzyme and MDA levels. Although no data were collected regarding the effect of ozone in blood enzymes, the dose used in this study was more that 100 times higher compared to ours (0.720mg/kg versus 0.063mg/kg). Furthermore, in our study ozone administration affected only GPx activity.

Chen *et al*., examine the hypothesis that ozone oxidative preconditioning exerts protective activity in renal ischemia/reperfusion injury associated with endogenous NO (Chen *et al.* 2008). Rats received 15 treatments with ozone (rectal insufflations) at a dose of 1 mg/kg, once a day. Control rats, that were not subjected to ischemia/reperfusion injury showed that ozone has no effect on the renal content of MDA, SOD, GSH and GPx. Intrarectal application of an ozone/oxygen mixture for 15 days at a dose of 1.1 mg/kg also fails to modify the SOD, catalase and GPx enzyme activity or the GSH concentration in the kidney (Borrego *et al.* 2004). The total amount of the ozone dose applied in the course of the experimental period was 63μg/kg (6.3μg/kg x 10 doses). Repeated application of ozone even in extremely small amounts resulted in significant changes to the antioxidant defense system capacity. Bearing in mind the impact of the selected scheme we could hypothesize that a "cascade" effect was possible evoked; repeated administration of ozone gradually attenuated blood's total antioxidant capacity. We can hypothesize that since superoxide dismutase levels remained stable and an increase was observed in GPx levels, a depletion of small non-enzymatic antioxidant molecules of blood was occurred; ascorbic acid, uric acid and probably albumin, who is characterized by its sacrificial behavior. Malondialdehyde levels in urine remained stable throughout the experimental period suggesting that the oxidative stress generated by ozone was handled adequately by the antioxidant system. Although TAC of RBCs, plasma and urine was decreased by almost 30%, it seems like the efficacy of the redox system was not compromised by this decrease. Thus, the elevation observed in GPx levels possibly acted as a protective mechanism against oxidant stress (either through *de novo* synthesis or by activation of metabolic pathways for defense).

The concentration of the antioxidant enzymes in the tissues varies to a great extent. Furthermore, there is a network of non enzymatic antioxidant that protects the various tissues from oxidative stress. Ozone effects on the enzymatic activity and the antioxidant capacity should be considered only for the specific tissue of interest and thus the doses applied should be not only route-dependent but also tissue-specific. Of course, ozone toxicity should be avoided by any cost.

One limitation of our study is that we did not monitor the activity of catalase; thus we cannot predict what the effect of ozone application to this enzyme was. Another limitation is that we treated the control group with NaCl and not with oxygen. Nevertheless, since medical ozone generators are widely used, ozone effects should be compared to untreated controls to define the net effects produced. On the other hand, the equipment used was highly accurate in setting and maintaining the chosen ozone concentration and flow-rate, and thus daily ozone application was well within the defined concentration.

CONCLUSIONS

Repeated intra-peritoneal application of extremely low doses of ozone resulted in an increase of GPx activity alongside with a deterioration of the total antioxidant capacity in RBCs, plasma and urine; without severely affecting the redox system, as indicated by the stable MDA concentration. Ozone doses must be adjusted to the different application methods and must be given in exact amounts as they can be either ineffective or toxic.

ACKNOWLEDGMENTS

The author gratefully acknowledges the assistance of Dr. Christodoulos Sioutis, bacteriologist for his assistance on blood analysis and Dr. Ioannis Zioris, chemist, for his technical and scientific assistance in LC-MS analysis.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

Altman N. 2007. How is ozone therapy applied? In: The oxygen prescription, 3rd edn. Rochester, Healing Art Press 44.

Bocci V. 2005a. How does ozone act? How and why can we avoid ozone toxicity? In: Ozone: A new medical drug. Netherlands: Springer 19.

Bocci V. 2005b. How does ozone act? How and why can we avoid ozone toxicity? In: Ozone: A new medical drug. Netherlands: Springer 27.

Bocci V. 2002. Oxygen-Ozone therapy A critical evaluation. Netherlands: Kluwer Academic Publishers.

Bocci V. The case for oxygen-ozonetherapy. Br J Biomed Sci, 2007; 64: 44-49.

Borrego A, Zamora ZB, Gonzalez R, Romay C, Menendez S, Hernandez F, Montero T and Rojas E. Protection by ozone preconditioning is mediated by the antioxidant system in cisplatin-induced nephrotoxicity in rats. Mediators Inflamm, 2004; 13: 13-19.

Calabrese E. Hormesis is central to toxicology, pharmacology and risk assessment. Hum Exp Toxicol, 2010; 29: 249-261.

Calabrese E. Hormesis and medicine. Br J Clin Pharmacol, 2008; 66: 594-617.

Charalampidis PS, Veltsistas P, Karkabounas S and Evangelou A. Blue CrO5 assay: A novel spectrophotometric method for the evaluation of the antioxidant and oxidant capacity of various biological substances. Eur J Med Chem, 2009; 44: 4162-4168.

Chen H, Xing B, Liu X, Zhan B, Zhou J, Zhu H and Chen Z. Ozone Oxidative Preconditioning Protects the Rat Kidney from Reperfusion Injury: The Role of Nitric Oxide. J Surg Res, 2008; 149: 287-295.

Hong YC, Lee KH, Yi CH, Ha EH and Christiani DC. Genetic susceptibility of term pregnant women to oxidative damage. Toxicol Lett, 2002; 129: 255-262.

Kesik V, Uysal B, Kurt B, Kismet E and Koseoglu V. Ozone ameliorates methotrexate-induced intestinal injury in rats. Cancer Biol Ther, 2009; 8: 1623-1628.

Lee HB and Blaufox MD. Blood volume in the rat. J Nucl Med, 1985; 26: 72-76.

Lindsay D. Nutrition, hormetic stress and health. Nutr Res Rev, 2005; 18: 249-258.

Noguchi N, Watanabe A and Shi H. Diverse functions of antioxidants. Free Radic Res, 2000; 33: 809-817.

Radak Z, Chung H and Goto S. Systemic adaptation to oxidative challenge induced by regular exercise. Free Radic Biol Med, 2008; 44: 153-159.

Sagai M and Bocci V. Mechanisms of Action Involved in Ozone Therapy: Is healing induced via a mild oxidative stress? Med Gas Res, 2011; 1: 29.

Shanker H. Hormesis and toxic torts. Hum Exp Toxicol, 2008; 27: 117-118.

Stark M. Hormesis, adaptation, and the sandpile model. Crit Rev Toxicol, 2008; 38: 641-644.

Zhang Y, Li S, Liang Y, Wen C, Guo Q and Su B. Potential mechanisms of neuroprotection induced by low dose total-body gamma-irradiation in C57 mice administered with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Neurosci Lett, 2009; 450: 106-110.

How to cite this article:

Yannis V Simos, Ioannis K Toliopoulos, Ioannis I Verginadis, Ilias V Karagounis, Pavlos S Charalampidis, Vasilios N Ragos, Angelos M Evangelou, Spiros C Karkabounas. Evaluation of intraperitoneal ozone application effects to rat's antioxidant enzymes, superoxide dismutase and glutathione peroxidase. J App Pharm Sci, 2013; 3 (11): 035-039.