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Selective ozone concentrations may reduce the ischemic damage after a stroke

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ORIGINAL ARTICLE

Selective ozone concentrations may reduce the ischemic damage after a stroke

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Abstract

Stroke is one of the most debilitating diseases, and it is unfortunate that only a small percentage of patients can be treated with thrombolytic agents. Consequently, there is an urgent need of finding an alternative procedure for reoxygenating the so-called *penumbra* at the earliest time as possible for reducing morbility and disability. A preliminary, preclinical study has been carried out by using rat hippocampal and cortical brain slices subjected to oxygen-glucose deprivation. Oxygen-ozone gaseous mixture appeared to be effective in reverting damage of brain tissues, supporting the evaluation of this approach in well-designed clinical trials in stroke patients.

Keywords: *ozone, brain ischemia, stroke, 4-hydroxy-2-nonenal*

Abbreviations: *ACSF, artificial cerebrospinal fluid; ARE, antioxidant response element; BSA, bovine serum albumin; GDH, glutamate dehydrogenase; HSA, human serum albumin; Keap1, Kelch-like ECH-associated protein 1; LDH, lactate dehydrogenase; MDA, malondialdehyde; NAD*$^+$*, β-nicotinamide adenine dinucleotide; NADH, β-nicotinamide adenine dinucleotide reduced form; Nrf2, nuclear factor [erythroid-derived 2]-like 2 (official symbol: NFE2L2); OGD, oxygen-glucose deprivation; PUFA, polyunsaturated fatty acids; OGD/R, oxygen-glucose deprivation and reoxygenation; ROS, reactive oxygen species*

Introduction

Among vascular diseases, stroke is caused by either atherosclerosis of a cerebral artery or embolism frequently due to atrial fibrillation. It is a frequent affection in aged patients, and it has a high rate of morbility, disability and mortality [1]. The prompt infusion of tissue plasminogen activators by triggering thrombolysis has shown considerable therapeutic activity in only about 5–10% of patients able to reach the stroke unit within 4.5 hours from the initial symptoms [2]. The remaining 90% of patients can be only carefully treated with anticoagulants, antihypertensive and antiaggregant drugs. Occlusion of a cerebral artery causes within 5–10 min an irreversible neuronal damage within the "core", while the peripheral tissue defined as the *penumbra* undergoes a less severe blood ischemia susceptible to be treated with a neuroprotective therapy [3]. Consequently, approaches to reduce the increase of glutamate, intracellular Ca^{2+}, formation of reactive oxygen species (ROS) as well as of proinflammatory cytokines leading to depolarization, inflammation and apoptosis are under intensive investigation [4–8] but valid therapeutic results remain elusive [9–11]. However, preliminary clinical studies [12,13], and anecdotal results (Wasser G, personal communication) have suggested that a

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prompt reoxygenation of the *penumbra* can be very beneficial.

Accumulating preclinical experiences with ozone administration for conditions associated with ischemia [14–16] support to perform clinical trials by evaluating the procedure of infusing twice daily ozonated autohemotherapy in patients who either reach the stroke unit after 4.5 hours or are over 80 years old. The rationale of this approach is not only of improving as soon as possible the oxygenation of the *penumbra* but also of inducing a number of metabolic modifications such as up-regulation of antioxidant enzymes, enhanced release of NO and CO as vasodilators and possibly a localized release of adenosine. However, as this trial requires a long preparation, we thought worthwhile to perform a preliminary, preclinical study about the role of a gaseous oxygen-ozone mixture as a potential therapeutic agent in stroke. A well-tested *in vitro* procedure with rat hippocampus and brain cortex slices subjected to ischemia-like conditions, that is, oxygen-glucose deprivation has been adopted [17–19]. Such *in vitro* model offers many advantages over *in vivo* techniques since it offers an immediate and direct access to the extracellular compartment due to the lack of a blood–brain barrier, and the environmental factors can be directly controlled. Furthermore, in brain slices the tissue morphology is relatively unchanged from the intact animal structure since intercellular connections are preserved, and this increases the likelihood that mechanisms of pathogenesis, which occur are representative of the *in vivo* situation [20].

Materials and methods

Compounds

Trizma® base, ascorbic acid, sodium pyruvate, β-nicotinamide adenine dinucleotide (NAD^+), β-nicotinamide adenine dinucleotide reduced form (NADH), glutamate, glutamate dehydrogenase (GDH), bovine serum albumin (BSA), thiobarbituric acid and all artificial cerebrospinal fluid (ACSF) components were acquired from Sigma–Aldrich Co. (St Louis, MO, USA). Human serum albumin (HSA) 4% was produced by Kedrion (Barga, Italy).

All other materials were from standard local sources and of the highest grade commercially available. Reagents were dissolved in MilliQ deionized water.

Animals

All experiments were performed in strict compliance with the recommendation of the EEC (86/609/CEE) for the care and use of laboratory animals, and the protocols were approved by the Animal Care and Ethics Committee of the University of Siena, Italy. Sprague–Dawley rats (350–450 g; Charles River Italia, Calco, Italy) were kept in large cages under a 12:12 hour day–night cycle at 20°C (ambient temperature). Drinking water and conventional laboratory rat food were available *ad libitum*. Before sacrifice, animals were anaesthetised by intraperitoneal injection of xylazine chloride (10 mg Kg^{-1}, Rompun® Vet., Bayer AG, Germany) and ketamine hydrochloride (35 mg Kg^{-1}, Ketavet®, Parke Davis/Warner–Lambert, USA).

Slices preparation

After sacrifice the whole brain was rapidly removed, chilled to 4°C and placed in artificial cerebrospinal fluid (ACSF) (composition in mM: 120 NaCl, 2.5 KCl, 1.3 $MgCl_2$, 1.0 NaH_2PO_4, 1.5 $CaCl_2$, 26 $NaHCO_3$, 11 glucose, saturated with 95% O_2–5% CO_2, with a final pH of 7.4, osmolality 285–290 mOsmol). The cortex was dissected and cut into 400 μm-thickness slices by using a manual chopper (Stoelting Co., Wood Dale, IL, USA). Afterwards, slices were maintained in oxygenated ACSF enriched with 400 μM ascorbic acid for 1 hour at room temperature to allow maximal recovery from slicing trauma [21].

In vitro *ischemia-like conditions*

During the initial 60 min, cortical slices % 4–5, total wet weight 33.6 ± 2.6 mg, n = 10) were placed in covered incubation flasks, containing 2 ml ACSF continuously bubbled with 95% O_2/5% CO_2 (Recover period) and incubated at 37°C for an additional period of 30 min (Equilibration). Afterwards, the phase of oxygen-glucose deprivation (OGD) was carried out by incubating slices for 30 minutes into covered incubation flasks, containing 2 ml ACSF but in which glucose was replaced by an equimolar amount of saccharose, and continuously bubbled with 95% N_2/5% CO_2. After the OGD period, ischemic solution was replaced by fresh, oxygenated 2 ml ACSF for an additional 90-minute period (Reperfusion). Treated samples were performed by adding increasing amounts of a gaseous mixture of oxygen/ozone or ozone to ACSF both in the absence and in presence of HSA (150 μg/ml) during the Reperfusion phase. The schedule already used [18,19] is reported in Figure 1.

Ozone generation

Ozone was generated from medical-grade oxygen using electrical corona arc discharge by Ozonosan PM 100K (Hansler GmbH, Iffezheim, Germany), which could deliver ozone concentrations up to 80 μg/ml with a gas flow ranging between 1 and 8 l/min.

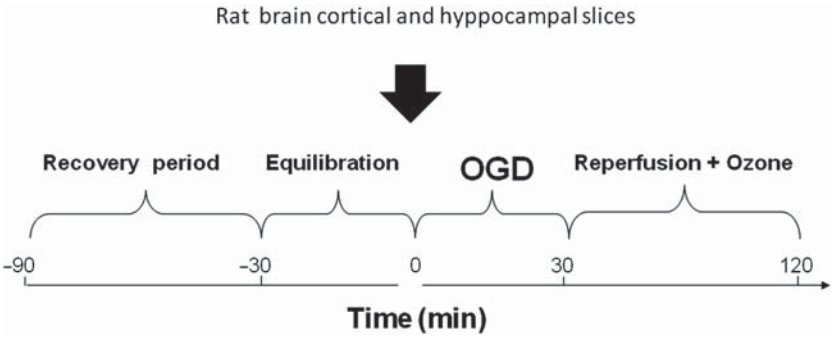

Figure 1. Scheme of the experimental protocol. Slices were maintained in ACSF continuously bubbled with 95% O_2/5% CO_2 for 1 hour at room temperature (Recovery period) and then equilibrated for an additional period of 30 minutes (Equilibration). Afterwards, the phase of oxygen/glucose deprivation (OGD) was carried out by incubating slices for 30 minutes in ACSF in which glucose was replaced by an equimolar amount of saccharose, and continuously bubbled with 95% N_2/5% CO_2. After the OGD period, ischemic solution was replaced by fresh, oxygenated 2 ml ACSF for an additional 90-minute period (Reperfusion).

In all cases, the ozone concentration was monitored continuously by photometry at 600 nm (Chappuis band), periodically checked by iodometric titration, as recommended by the Standardisation Committee of the International O_3 Association. The photometer was periodically checked by using the iodometric titration in observance of the rules established by International Ozone Association (IOA). Medical oxygen and unfiltered air has been used because the latter contains 78% of nitrogen with the inherent formation of nitrogen oxides.

Single-use silicon treated polypropylene syringes (ozone-resistant) and tygon polymer tubing were used throughout the procedure to ensure containment of ozone and consistency in concentrations [22].

Assessment of neuronal injury

Neuronal damage was assessed quantitatively by measuring the amount of both glutamate and lactate dehydrogenase (LDH) released into the ACSF during 90-minutes-reperfusion period. In particular, glutamate was measured fluorimetrically (excitation 366 nm; emission: 450 nm) using the conversion of NAD^+ to NADH by glutamate dehydrogenase [17]. For glutamate release, data are expressed as nmol/mg wet tissue.

As a marker of tissue damage, LDH released from the slices was determined spectrophotometrically by the rate of decrease in absorbance at 340 nm via the oxidation of NADH to NAD^+ using pyruvate as substrate [17]. Results were calculated as U/mg wet tissue (one unit of LDH activity is defined as that, which gives rise to one micromole of lactate in 1 minute) and expressed as a percentage of that released by control slices.

Tissue water gain (edema) was calculated as described by MacGregor et al. [23]. Briefly, at the end of experimental session, slices were weighed on preweighed pieces of aluminium foil (typically 2×2 cm), dried overnight at 95°C and then reweighed. All water content data were referred to tissue dry weight, which is assumed to be constant under conditions of water gain [24]. Finally, to quantify lipid peroxidation in brain slices under basal conditions and after oxygen-glucose deprivation and reoxygenation (OGD/R), method of De La Cruz et al. was used [25]. The results were expressed as µmol of malondialdehyde (MDA) formed per milligram of protein, the latter determined with the method of Lowry et al. [26].

Data analysis

The experiments were performed by using brain slices derived from at least three rats. Data are reported as mean ± S.E.M., and "n" is defined as the number of samples.

Statistical analysis was performed by using one-way ANOVA followed by Tukey–Kramer post-test or one sample t-test, as appropriate (GraphPad Software, San Diego, CA, USA). In all comparisons, the level of statistical significance (p) was set at 0.05.

Results

The results are reported in Figures 2–4. In detail, rat cortical slices incubated in ACSF for 120 min period (control conditions, CTRL) were found to release 2.25 ± 0.21 U/mg (value used as 100%, n = 26) (Figure 2, panel A) and 0.18 ± 0.02 nmol/mg tissue (n = 16) (Figure 2, panel B) of LDH and glutamate, respectively. Water content of the CTRL for evaluating edema conditions was 7.31 ± 0.21 g H_2O/g dry weight (Figure 2, panel C). As expected, 30 minutes of oxygen-glucose deprivation followed by 90 minutes of reperfusion (OGD/R) caused a highly significant release in LDH and glutamate release as well as tissue edema with respect to CTRL ($p < 0.001$). Ozone (20–160 µg/ml) antagonized OGD/R-induced LDH and glutamate release. This antagonism, however, followed a "U-shaped" concentration-response curve, typical of a hormetic behaviour. In particular,

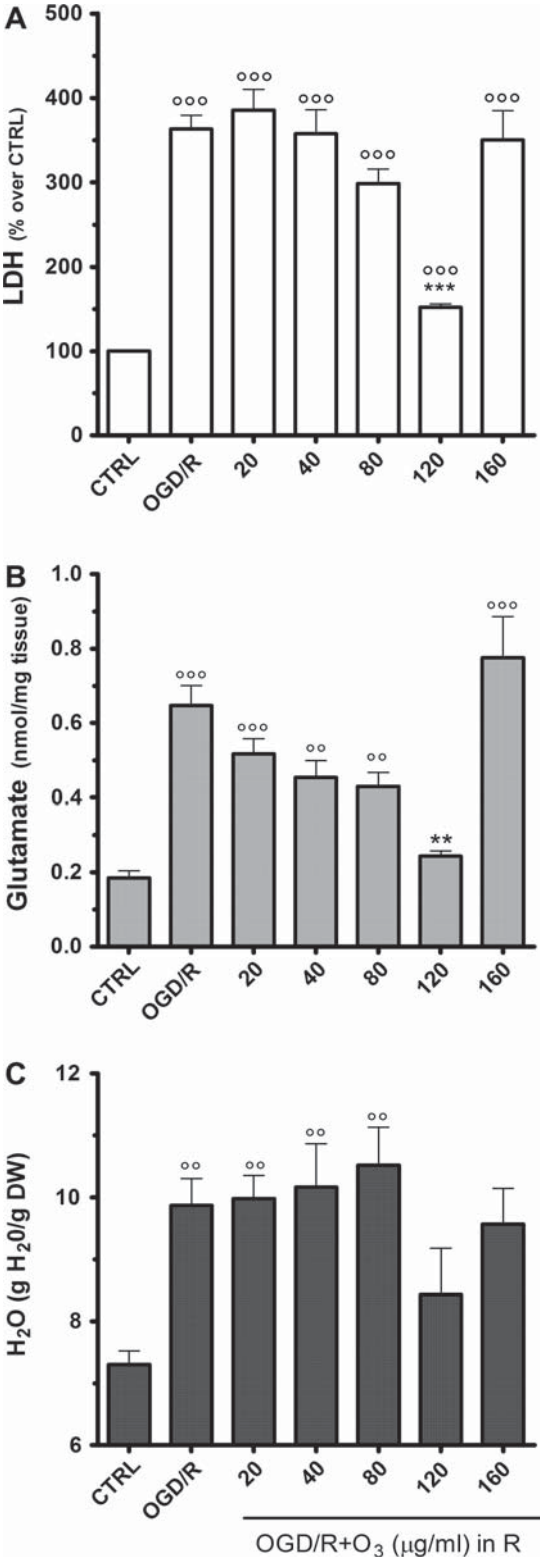

Figure 2. Effects of ozone on oxygen-glucose deprivation and reoxygenation-induced release of glutamate (panel A), LDH (panel B) and on tissue water content (panel C) in rat brain slices. Slices were incubated in ACSF for 120 minutes (control conditions, CTRL) or subjected for 30 minutes to oxygen/glucose deprivation followed by 90-minute immersion in normally oxygenated ACSF (OGD/R). Increasing concentrations of ozone (20–160 μg/ml) were added to ACSF during reperfusion. Data are means ± S.E.M. of at least four different experiments. °°$p < 0.01$ °°°$p < 0.001$ vs CTRL; **$p < 0.01$, ***$p < 0.001$ vs OGD/R.

120 μg/ml proved to be the successive reperfusion phase of 120 minutes most effective concentration, as LDH efflux into ACSF amounted to 151.8 ± 4.4 % ($p < 0.001$ vs OGD/R, n = 12) and that of glutamate efflux into ACSF to 0.24 ± 0.01 nmol/mg tissue ($p < 0.01$ vs OGD/R, n = 12), restoring values comparable to those of CTRL. Lower (20–80 μg/ml) or higher (160 μg/ml) ozone concentrations had not statistically significant effects. On the contrary, as to tissue edema not one ozone concentration significantly reduced this parameter ($p > 0.05$ vs OGD/R, n = 10–12), although water gain in slices treated with 120 and 160 μg/ml was not different from that found in CTRL ($p > 0.05$ vs CTRL, n = 10–12) (Figure 2, panel C).

Improved results are shown in Figure 3. In this case also the ozone concentration of 80 and 120 μg/ml have been able to significantly reduce ($p < 0.001$ vs OGD/R, n = 10–12) the extruded LDH and glutamate (Figure 3, panel A and B, respectively), restoring values comparable to those of CTRL. This different behaviour appears to be due to the addition of HSA (150 μg/mL) to the ACSF used during reperfusion phase. As for edema, it was significantly reduced ($p < 0.05$ vs OGD/R, n = 12) only in samples treated with 120 μg/ml ozone (Figure 3, panel C). Interestingly, as it can be observed in Figure 4, even in the presence of HSA to the ACSF, OGD/R-induced formation of MDA was reduced to values comparable to controls in slices containing ozone at the concentration of 40 μg/ml ($p < 0.05$ vs OGD/R), 80 μg/ml ($p < 0.01$ vs OGD/R) and 120 μg/ml ($p < 0.05$ vs OGD/R).

Discussion

Data from the literature suggests the use of ozone therapy in ischemic and hypometabolic brain syndromes such as stroke. Consequently, the aim of the present study was to assess the effects of ozone in an *in vitro* model of brain ischemia based on rat brain slices subjected to OGD and reperfusion, which allows to test a wide range of ozone concentrations in the same experimental setting. The present results demonstrate that ozone, when present in the reperfusion medium with HSA, was very effective in reverting OGD and reperfusion-induced damage of brain tissues. Its neuroprotective effect, however, was related to the concentration according to an "U" shaped curve, typical of an hormetic phenomenon, with an efficacy windows being displayed in a concentration range of 80–120 μg/ml. Quite often the concentration/response relationship of many drugs is characterized by a hormetic behaviour [27]. In the case of ozone, a possible explanation of this effect could lie in a sort of "preconditioning response" often leading to both a repair and an increased

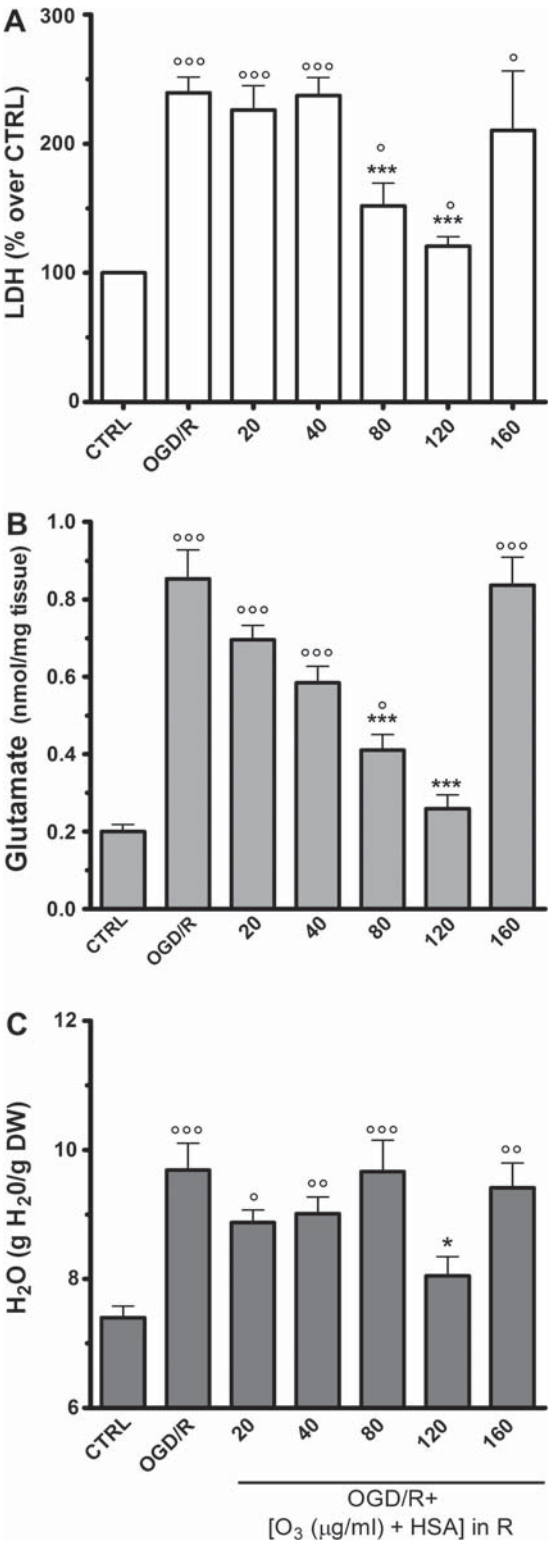

Figure 3. Effects of Ozone and Human Serum Albumin (HSA) on oxygen-glucose deprivation and reoxygenation-induced release of glutamate (panel A), LDH (panel B) and on tissue water content (panel C) in rat brain slices. Slices were incubated in ACSF for 120 minutes (control conditions, CTRL) or subjected for 30 minutes to oxygen/glucose deprivation followed by 90 min immersion in normally oxygenated ACSF (OGD/R). Increasing concentrations of ozone (20–160 μg/ml) were added during reperfusion to ACSF containing 150 μg/ml of HSA. Data are means ± S.E.M. of at least four different experiments. °$p < 0.05$, °°°$p < 0.001$ vs CTRL; *$p < 0.05$, ***$p < 0.001$ vs OGD/R.

defence capacity well within the "overcompensation stimulation hormesis" [28].

By considering that using *in vitro* models only the slice surfaces and the inner superficial tissue layers can take advantage of the normal ACSF without or with albumin, the results are surprising and need to be interpreted. Previous data on chronic limb ischemia [29] by using human blood gassed with oxygen-95%-ozone-5% showed that oxygen simply hyperoxygenates the plasma and fully oxygenates haemoglobin, while ozone acts as a pro-drug. Indeed, ozone, which more rapidly and to a greater extent dissolves in the aqueous environment of plasma with respect to oxygen, immediately reacts with solutes absent in the ACSF, especially hydrosoluble antioxidants (uric acid, ascorbic acid, trace of GSH), and with polyunsaturated fatty acids (PUFA) bound to albumin, when present. In the presence of water, the very rapid reaction yields 1 mol of H_2O_2 and 2 mol of alkenals, of which 4-HNE is the major component. Hypothetically, the adducts Cys34 of albumin with 4-HNE can bind to the complex Nrf2 (nuclear factor erythroid 2-related factor 2)/Keap1 (Kelch-like ECH-associated protein 1) in the cytoplasm. The released Nrf2 enters into the nucleus and bind to the antioxidant response element (ARE) and induces the transcription of various antioxidants and phase II detoxifying enzymes [30–33].

In retrospect, ozone dosages of 80–120 μg/ml resemble the optimal ratio between 1 ml of blood

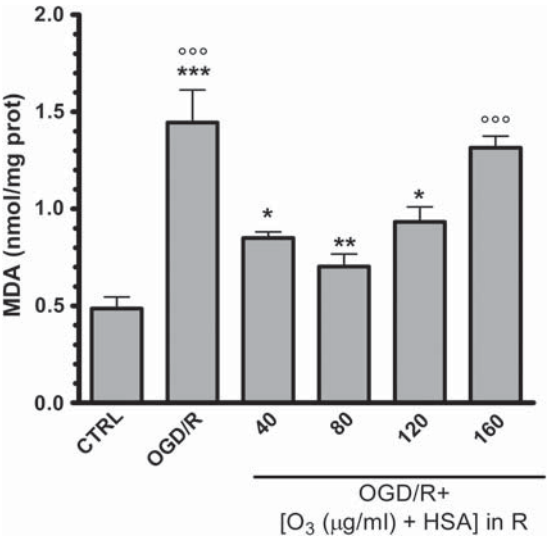

Figure 4. Effect of ozone on oxygen-glucose deprivation and reoxygenation-induced release of MDA in rat brain cortical slices. Slices were incubated in ACSF for 120 minutes (control conditions, CTRL) or subjected for 30 minutes to oxygen/glucose deprivation followed by 90-minute immersion in normally oxygenated ACSF (reoxygenation). Increasing concentrations of ozone (20–160 μg/ml) were added during reperfusion to ACSF containing 150 μg/ml of HSA. Data are means ± S.E.M. of at least four different experiments. °°°$p < 0.001$ vs CTRL; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs OGD/R.

(about 600 µL of plasma) and the repeatedly determined useful ozone dosages ranging from 20 to 80 µg ozone dissolved in 1 ml of blood [34]. Consequently, it can be envisaged that *in vitro* brain tissue, at least in part, enters in contact with both 20–40 µmol of H_2O_2 and submicromolar concentrations of alkenals. Both are able to diffuse within the brain tissue and reverse the damage occurred during the previous "ischemic period" or "oxygen-glucose deprivation" 30-minute period. In regard to blood, rational concentrations of both H_2O_2 and alkenals have shown to be able to trigger a number of useful biochemical reactions leading to therapeutic effects after infusion of ozonated blood into the donor patient [35], keeping in mind that in patients ozonation of blood must be performed only using whole blood and the appropriate ozone concentrations in order to exclude any cell damage or toxicity [22,36]. Concisely, these effects can be summarized as: i) improved release and delivery of oxygen and glucose; ii) up-regulation of intracellular antioxidant enzymes following the calculated and precise oxidation stress and iii) a feeling of wellness in patients. In conclusion, these *in vitro* results are the first to reveal the neuroprotective effects of ozone in an *in vitro* model of brain ischemia and encourage to perform a clinical trial in stroke patients not eligible for thrombolytic treatment.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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