

Index

■ Editorial	3		
M. Muto			
■ Original Articles			
PERCUTANEOUS TREATMENT OF LUMBAR DISC HERNIATION BY OXYGEN-OZONE INJECTION A CLINICAL STUDY OF 322 CASES	6	OZONE CHEMYNUCLEOLYSIS VS MICRODISCECTOMY. PROSPECTIVE CONTROLLED STUDY WITH 18 MONTHS FOLLOW-UP	49
Wang Zhu Ying, Jiang Cai Mei, Wang Zhi Min		J. Buric	
USE OF FAT/SAT MAGNETIC RESONANCE SEQUENCES WITH GADOLINIUM IN THE PRE-TREATMENT AND FOLLOW-UP ASSESSMENT OF PATIENTS UNDERGOING OXYGEN-OZONE THERAPY	9	OXYGEN-OZONE THERAPY: CT -GUIDED INTRAFORAMINAL AND PERIGANGLIONIC INFILTRATION. PERSONAL CASE SERIES AND LITERATURE REVIEW	55
M. Bonetti, A. Fontana		G. Venza, A. Marrone	
O ₂ -O ₃ THERAPY IN TENDINOPATHIES AND ENTRAPMENT SYNDROMES	20	OXYGEN-OZONE THERAPY IN SPORT. A CASE REPORT	59
B. Moretti, R. Lanzisera, G.L. Sisti, L. Moretti, S. Patella, V. Patella, C. Simone		L. Re	
THE USE OF HYDROGEN PEROXIDE AS A MEDICAL DRUG	30	PARAVERTEBRAL O ₂ -O ₃ TREATMENT IN MECHANICAL LUMBAR PAIN IN RIDING HORSES	64
V. Bocci, Aldinucci, L. Bianchi		A. Vigliani, E. Boniperti, E. Scudo	
NEW DATA REGARDING THE USE OF OXIDATIVE STRESS (OZONE THERAPY) IN THE FORMER SOVIET UNION COUNTRIES	40	OXYGEN-OZONE THERAPY FOR SPINAL MUSCLE DISORDERS IN THE HORSE	70
S. Ikonomidis, P. Tsaousis, A. Fyntanis, EM. Iliakis		E. Ballardini	
TOTAL CLINICAL AND RADIOLOGICAL RESOLUTION OF ACUTE, MASSIVE LUMBAR DISC PROLAPSE BY OZONUCLEOLYSIS THE FIRST INDIAN CASE REPORT	44	■ Letters to the Editor	74
V.S. Kumar		■ Intermezzo	78
		TYPICAL SPECIES FROM PUERTO RICO'S RAIN FOREST	
		■ FIO - Newsletter	79
		■ FIO - Patronages received	94
		■ Information & Congresses	98, 99, 100, 101, 102, 103, 104, 105, 106, 107
		■ Subscription Form	108
		■ FIO Application Form	109
		■ Instructions to Authors	112

Cover: Lizard endemic to the rain forest of Puerto Rico
(Photo by M. Bonetti)

The Use of Hydrogen Peroxide as a Medical Drug

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Key words: hydrogen peroxide, ozone, ROS, LOPs, oxidative stress, macular degeneration

SUMMARY – Since 1993, a diluted solution of hydrogen peroxide (H_2O_2) has been administered intravenously in many ischaemic and neoplastic patients in the USA and Canada as a bio-oxidative therapy. However, to date no biochemical study has defined the behaviour and fate of H_2O_2 diluted in either human blood or a 5% glucose solution. Our study aimed to define the stability of H_2O_2 *in vitro* and *in vivo* and to compare the generation and stability of reactive oxygen species (ROS) and lipid oxidation products (LOPs) when blood is treated with either ozone or H_2O_2 . In addition, we evaluated the therapeutic effect of the gluco-peroxide solution in age-related macular degeneration (ARMD) patients with encouraging results. There is a general consensus that H_2O_2 is one of the most important physiological messengers and because it could activate multiple cell targets, H_2O_2 could become a useful medical drug particularly in countries with scant medical resources.

Introduction

During the last 35 years reactive oxygen species (ROS) have come of age and, if not the direct cause of several human pathologies, they certainly maintain chronic oxidative stress. More recently the concept that cells can regulate redox-sensitive signal transduction pathways and transcriptional regulatory processes through a physiological generation of ROS has gained a wide consensus. Needless to say, excessive and continuous release of ROS during chronic inflammation are partly responsible for cell degeneration and death.

Although hydrogen peroxide (H_2O_2) is not a radical molecule, it has oxidizing properties and can act as a second messenger in several biological processes. H_2O_2 modulates protein phosphorylation through cysteine oxidation of nuclear factor κB (NF κB)¹, AP-1², and T-cell serum response factor[3] are all involved in ROS signalling mediated by hydrogen peroxide. A fairly low concentration of H_2O_2 (121 microMole) stimulates fibroblast proliferation⁴, neoangiogenesis^{5,6} and activates lymphocytes⁷. H_2O_2 can also stimulate IL-8 production in leukocytes^{8,9} and in cultured endothelial

cells to a level that can induce angiogenesis¹⁰. Moreover it is important for the transduction of signals by PDGF¹¹, insulin¹², leukotriene B4¹³ and TNF-alpha¹⁴. We found that ozonation of human plasma triggers a reaction leading to release of H_2O_2 and lipid oxidation products (LOPs)^{15,16} so that ozonated serum briefly added to human endothelial cells in culture induces a significant and steady increase in nitric oxide (NO) production¹⁷. Interestingly, addition of H_2O_2 (20-40 microMole) to the same cell system rapidly increases the release of NO, that is further potentiated by L-Arginine (20 microMole) addition and totally blocked by the NO synthase inhibitor NG-nitro-L-arginine methyl ester. Moreover, Brown¹⁸ and Thengchaisri and Kuo¹⁹ have suggested that H_2O_2 induces endothelium-dependent vasodilation through activation of cyclooxygenase-dependent prostaglandin E_2 formation. Hydrogen peroxide can be produced inside the cell and also in the extracellular space by a membrane bound NADPH oxidase. It readily diffuses through the plasma membrane and acts as a second messenger inside the cell. "Physiological" concentrations of H_2O_2 can trigger several biochemical pathways (reviewed in Bocci^{15,16} without

deleterious effects because the normal cell has an efficacious antioxidant capacity that rapidly reduces it to water. When H_2O_2 is produced extracellularly or added to a cell culture system a gradient of H_2O_2 is rapidly established across the plasma membrane²⁰. Stone and Collins²¹ demonstrated that this gradient is the result of H_2O_2 -scavenging enzymes (catalase, GSH-peroxidase) and results in the steady-state intracellular concentration being about tenfold less than the extracellular concentration. This result is important because the intravenous (IV) infusion of a low and calculated concentration of H_2O_2 results in a marked dilution in the plasma pool with partial inactivation and in intracellular levels able to exert biological effects on blood and endothelial cells without aggravating the concomitant oxidative stress. In 1888 Love²² perceived that "hydrogen peroxide could be used as a topical remedial agent". He had a wonderful insight into a problem that was clarified only some eighty years later showing that phagocytes can only defeat pathogens when they deliver ROS (O_2^- , H_2O_2 , HOCl and NO)²³. Only in 1993 did Farr²⁴ promote the intravenous (IV) administration of an aqueous solution of H_2O_2 diluted in a isotonic glucose in a few illnesses and founded bio-oxidative therapy, included by the NIH (Bethesda, MD, USA) among the complementary medical approaches. Clinical studies in ischaemic and neoplastic patients have subsequently been reported by Urschel²⁵, Sasaki²⁶, Nathan and Cohn²⁷ and Symons²⁸. However no scientific evaluation on the stability and pharmacokinetics of H_2O_2 and derivatives has been carried out and we present our results.

Materials and Methods

Materials

Analytical grade H_2O_2 30% was purchased from Fluka GmbH, Switzerland and stored in the cold room. Twice crystallized, aqueous solution catalase from bovine liver and all the materials not specified below were purchased from Sigma Aldrich srl, Milan, Italy. Anticoagulants for human use were either heparin (calcium salt) or 3.8 % sodium citrate solution (1 ml per 9 ml of blood). Sterile apyrogenic glucose solution 5% in 250 ml glass bottles was purchased from Galenica Senese, Siena, Italy.

Ozone generation and measurements

Ozone was produced from medical grade oxygen using electric corona arc discharge with a modern ozone generator (Ozonosan PM 100K,

Hansler GmbH, Iffezheim, Germany) which allows the gas flow rate and ozone concentration to be controlled in real time by photometric determination as recommended by the Standardization Committee of the International Ozone Association. Tygon tubing and ozone-resistant disposable syringes were used throughout the reaction procedure to ensure containment of ozone and consistency in concentration.

Ozone delivery to human blood

A predetermined volume (usually 5 ml) of oxygen-ozone gas mixture at various ozone concentrations within the therapeutic range (20-80 $\mu\text{g/ml}$ of gas per ml of blood or plasma) was collected with a syringe and immediately introduced into a second, 10 ml syringe, via a multidirectional stopcock containing an identical volume of either plasma or blood samples obtained from normal donors at the Siena Clinical Blood Centre. The final gas pressure remained at normal atmospheric pressure. Ozone is a very reactive gas so that rapid and precise handling is required to ensure reproducible results. In order to avoid foaming, samples were gently but continuously mixed with the gas phase for ten minutes, i.e. the period of time allowing the total reaction of ozone with blood. Afterwards they were dispensed into test tubes for analyses. Control samples were either used as such or mixed with an equal volume of oxygen. This control is necessary because oxygen accounts for at least 96% of the gas mixture. Ozonated autohaemotherapy (AHT) was performed¹⁵ by adding 225 ml of blood into a neutral glass sterile bottle under vacuum to 25 ml of Na citrate (3.8%) solution. Next, 225 ml of gas mixture containing ozone at a concentration of 40 $\mu\text{g/ml}$ per ml of blood (total dose: 9 mg) were added and, after the usual ten minutes gentle mixing, the oxygenated-ozonated blood was reinfused during the following 20 min into the donor (one of us, VB, has volunteered to do these autotransfusions since 1995). To evaluate the pharmacokinetics of thiobarbituric acid reactive substances (TBARS) and protein thiol groups (PTG) plasma level values, small blood samples were withdrawn before, at ten and 20 min infusion period and then at two, ten, 20 and 30 min during the post-perfusion period.

Preparation of the glucose solution containing hydrogen peroxide (gluco-peroxide solution)

Using a sterile hood, a hydrogen peroxide solution at 15 % is prepared by diluting 30% reagent grade H_2O_2 with an equal volume of apyrogenic s-

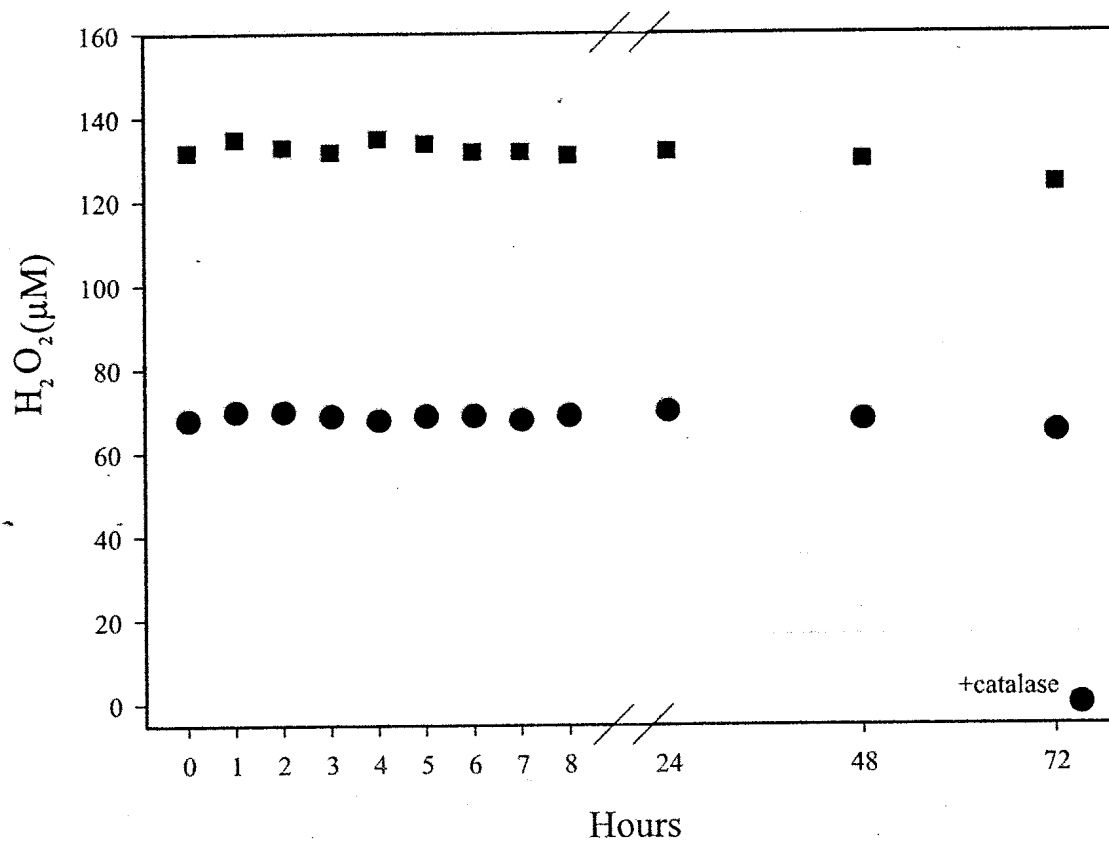


Figure 1 Stability of two solutions (68 and 132 microMole) of H_2O_2 , kept at $+21^\circ C$ in 5% glucose solutions. Average of two determinations.

terile bidistilled water. The final solution is prepared by injecting 0.5 ml of the 15% H_2O_2 solution into the 250 ml flask of 5% glucose solution via a sterile mini-spike plus particle filter (Ref. 4550234, B. Braun, Melsungen, Germany). During preparation, either glass or polypropylene disposable sterile syringes are recommended, avoiding the use of a metal needle.

The final H_2O_2 concentration is equivalent to 0.03% (8.8 mM) and the solution is isotonic and suitable for direct slow (20 min) IV infusion via a G-23 or a G-25 angiocath (plastic catheter). H_2O_2 should never be diluted into saline to avoid the risk of HOCl formation.

The H_2O_2 solution for topical use has a concentration of 3.5% (12 volumes). According to Farr²⁴, the final H_2O_2 concentration in the gluco-peroxide solution can vary from 0.03% (8.8 mM) to a maximum of 0.15% (44 mM) which must be infused at a slow rate.

The pharmacokinetic study presented here was carried out in duplicate using a 0.12% (35.2 mM) concentration infused within 20 min without any side effects (VB volunteered for the analysis). While we followed Farr's formulation, we re-

frained from adding both Mg and Mn chloride which may alter H_2O_2 stability.

Therapeutic effect of the gluco-peroxide solution in ARMD patients.

Owing to a lack of suitable venous accesses for blood collection, women with ARMD have the only option to be treated with the gluco-peroxide solution via a small, visible vein in the back of a hand. After being fully informed about this new experimental therapy and signing an informed consent form, this therapy has been so far performed in six women (age: 71 ± 11) at the "Misericordia Medical Clinic" Taverne d'Arbia, Siena by VB as a physician. In order to induce tolerance, we adopted the strategy: start low, go slow. Thus H_2O_2 concentration in the glucose solution was 0.03% for the first week (two treatments), then 0.06% for the second week, 0.09% for the third week and finally 0.12% for the fourth and following three weeks. Results are reported in Section 6. Provided that the solution is injected slowly, even the final concentration does not cause any venous irritation.

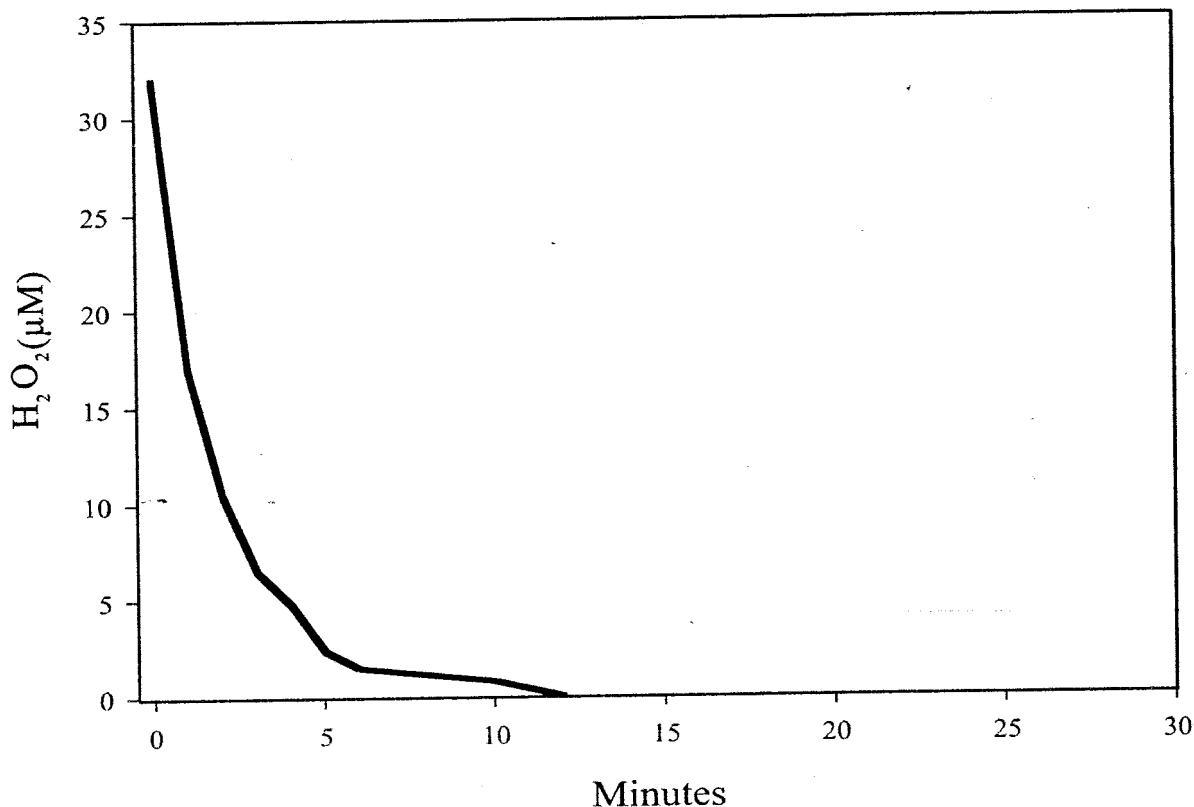


Figure 2 Rapid decay at 21 °C of H₂O₂ diluted in human plasma. The half-life is about 1 min.

Biochemical determinations

- Hydrogen peroxide was measured in the gluco-peroxide solution and human plasma by the enzymatic method described by Green and Hill²⁹. When necessary, catalase (20-40 Units) was added to the solution under test.
- Total antioxidant status (TAS) in plasma samples was determined according to Rice-Evans and Miller³⁰. Values are reported in mM terms.
- The thiobarbituric acid assay completed with butanol extraction was carried out in plasma as described by Buege and Aust³¹. Values are expressed as microMole of TBARS relative to a malonyldialdehyde (MDA) standard.
- PTG were measured in plasma according to Hu³² using procedure 1 with 5,5'-dithio-bis(2-nitrobenzoic acid, DTNB) dissolved in absolute methanol. Values are reported as mM.
- The haemoglobin determination was performed using 20 microLitres of original blood and an equal volume of plasma collected after the ozonation. Samples were mixed with 5 ml of the cyanide-methaemoglobin reagent (Sclavo Haemoglobin test kit). Optical density, read spectrophotometrically at 540 nm, was converted to

haemoglobin according to a standard curve and referred to as a percentage of total haemoglobin.

Statistical analysis

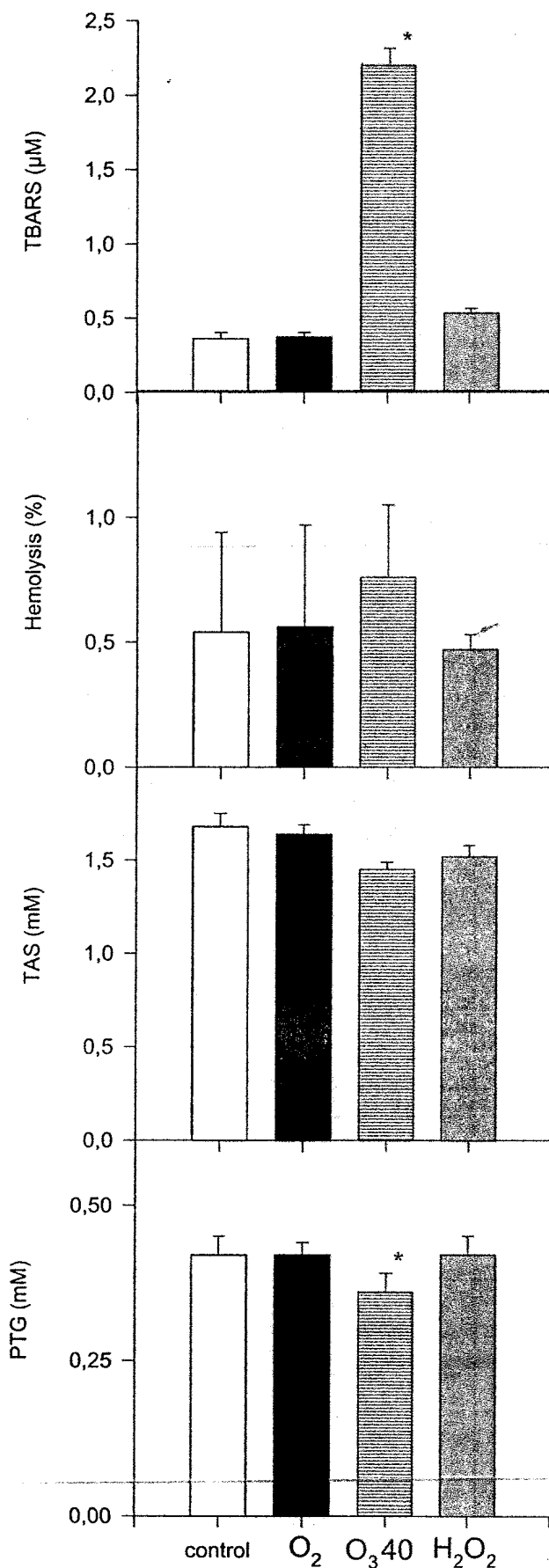
Whenever possible, results were expressed either as an average of two determinations or as means \pm SD. Statistical evaluation of the experimental data was performed with Student's *t*-test for paired samples with ≤ 0.05 as the minimal level of significance ($P \leq 0.05^+$).

Results

1) Stability of the gluco-peroxide solution

Figure 1 shows that the repeated titration of two solutions at different H₂O₂ concentrations, kept at +21°C, shows no practical change in concentrations up to three days. The final addition of catalase lowers the titres to zero within a few minutes. A sample of the H₂O₂ solution (68 microMole) kept at +4°C remains equally stable for three days (data not shown). The gluco-peroxide solution, prepared in the late morning is infused in patients

Figure 3 Three human blood samples were exposed to air (control) or O₂, or O₂-O₃ with an ozone concentration of 40 mcg/ml or to H₂O₂ (0.12%) for 1 min. TBARS, TAS and PTG levels varied significantly (+P<0.05) only after ozone exposure.



in the afternoon or otherwise discarded. For current medical use the hospital and private pharmacists are qualified to preparing the solution.

2) *Instability of hydrogen peroxide in human plasma*

Addition of the gluco-peroxide solution to human plasma anticoagulated with Na citrate leads to a very rapid disappearance of the H₂O₂ titre. The half-life is estimated to be about one min (figure 2). The reduction of hydrogen peroxide is mainly due to the presence of physiological amounts of uric and ascorbic acids and traces of antioxidant enzymes (catalase and GSH-peroxidase). Addition of both 3-amino-1,2,4-triazole and N-ethylmaleimide delays but does not block the disappearance of H₂O₂.

Addition of the gluco-peroxide solution to human blood leads to such an extremely rapid disappearance of the H₂O₂ titre that it is technically impossible to detect it even 90 s after addition. Besides the presence of antioxidants, the erythrocyte mass mops up H₂O₂ almost instantaneously.

3) *Peroxidation of human blood by either ozone or H₂O₂*

Three human normal blood samples (5 ml each) anticoagulated with Na citrate were either treated

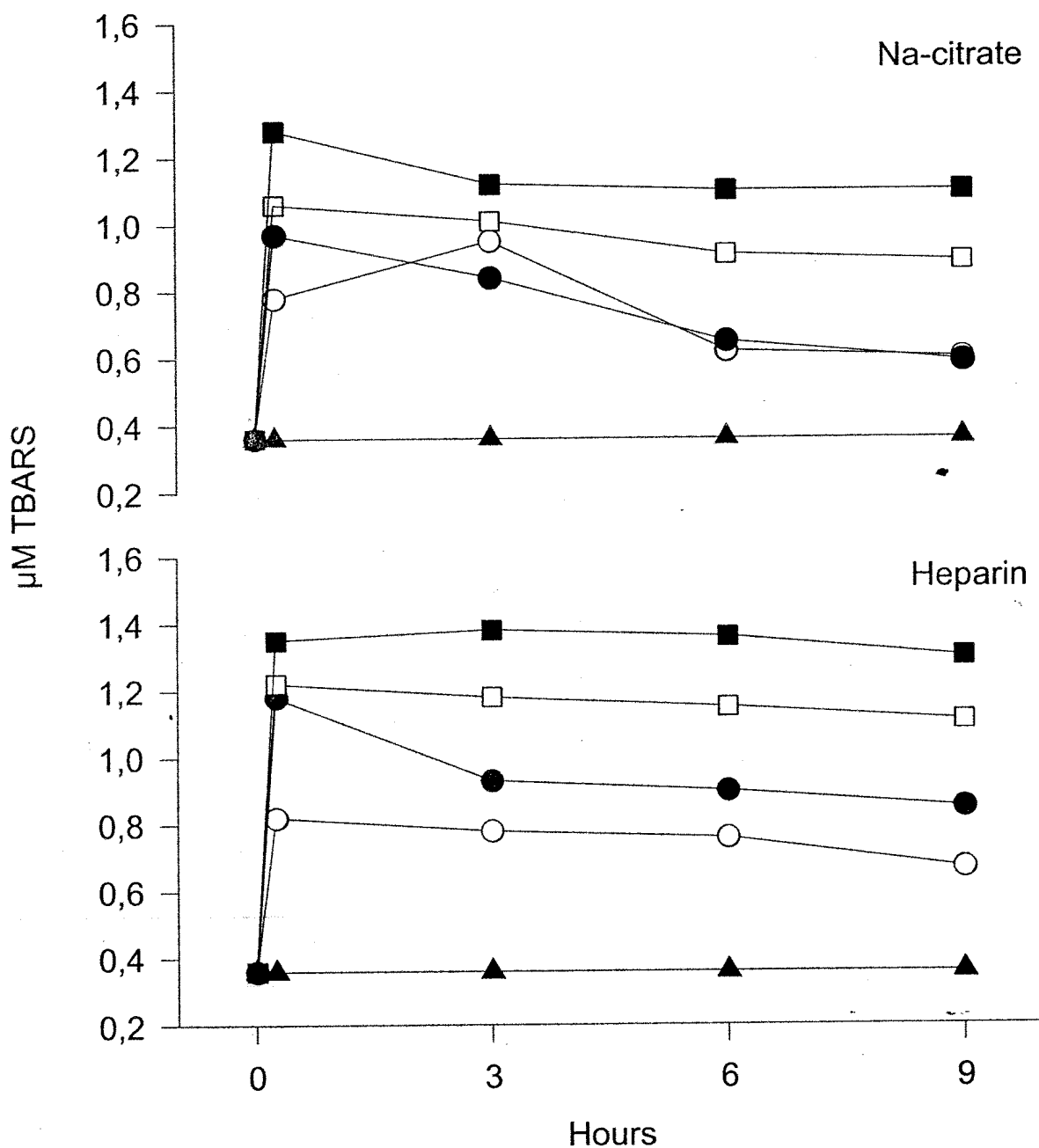


Figure 4 Kinetics of TBARS mean values measured in two normal human plasma samples before (0 time) and immediately after ozonation up to 9 hours with ozone concentrations of 10 (○), 20 (●), 35 (□) and 70 (■) mcg/ml per ml of blood. Controls are samples exposed to O₂ only (▲). All samples were incubated at +37 °C.

with 5 ml gas containing an ozone concentration of 40 mcg/ml (total dose: 0.2 mg ozone) or supplemented with a gluco-peroxide solution so that the final concentration of H₂O₂ was 0.06% (17.6 mM).

Figure 3 compares the peroxidation (TBARS), haemolysis, total antioxidants (TAS) and protein oxidation (PTG) values measured after addition of either ozone or H₂O₂. Within the previously defined therapeutic window^{15,16}, the ozone concentra-

tion of 40 mcg/ml per ml of blood is in the middle range and yet, in comparison with control and simply oxygenated samples, it appears to modify the blood sample significantly more than the gluco-peroxide solution. Indeed the generation of LOPs is far higher after ozonation than after H₂O₂ addition and this was expected as H₂O₂ is only one compound of the many generated during the reaction of ozone with blood components.

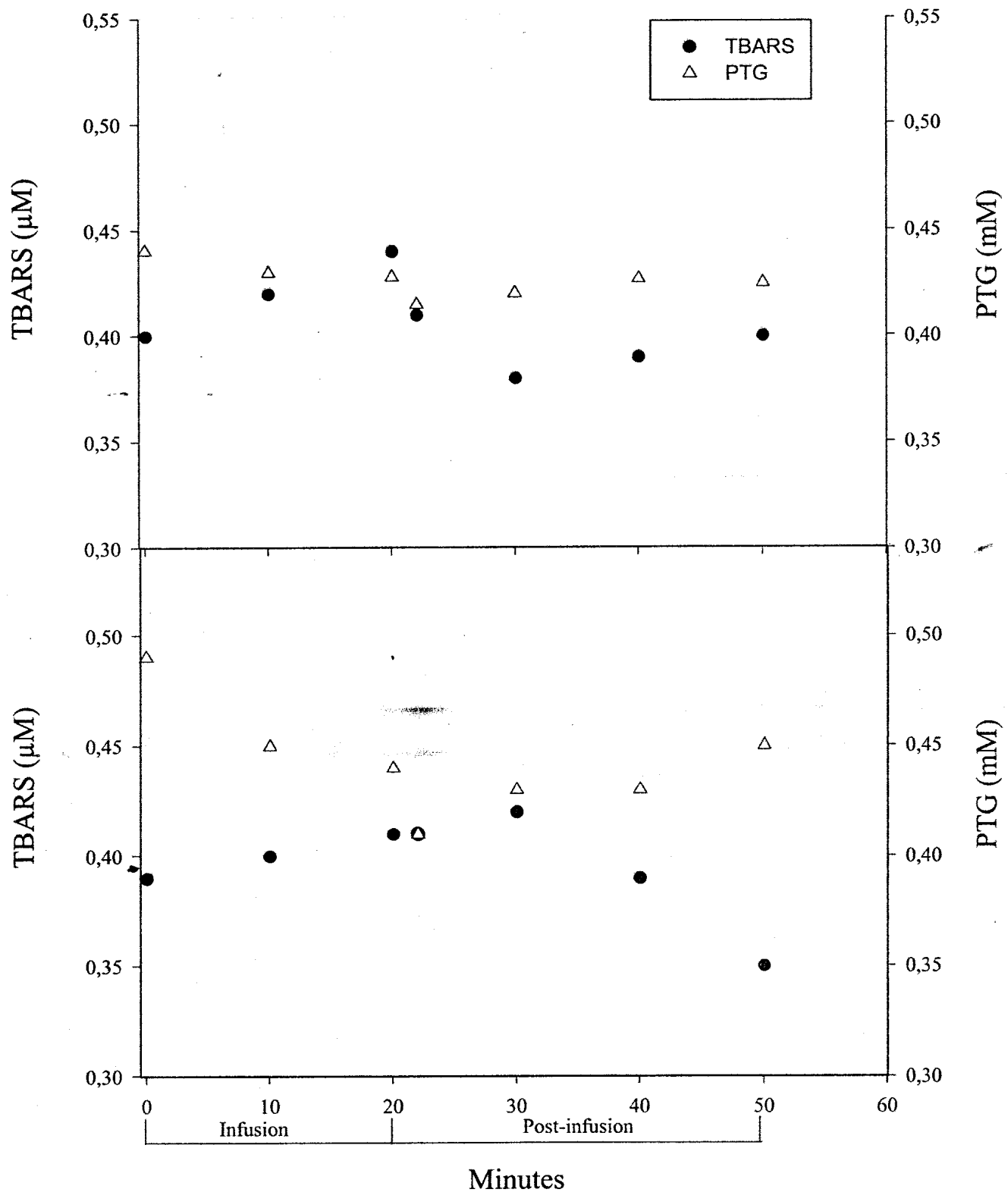


Figure 5 A e B Kinetics of LOPs *in vivo*. (A) In the case of ozonated blood, TBARS levels in plasma increases slightly only during the infusion period and thereafter they fluctuate within normal values. (B) The infusion of the gluco-peroxide solution does not modify TBARS levels. In both cases, PTG values are hardly modified.

4) Stability of LOPs *in vitro*

H₂O₂ as a typical marker of ROS disappears very rapidly from body fluids either after its addition or after ozonation of human plasma *ex vivo*. On the other hand, LOPs (a heterogeneous mixture of MDA and alkenals) appear to be stable *in vitro*, even when incubated at +37°C at pH 7.3. Figure 4 shows that different concentrations of these compounds decay slowly *in vitro* and this result helps to explain why they are toxic in static tissue cultures even at 1 microMole concentration.

5) What is the fate of LOPs *in vivo*?

A preliminary result³³ has now been amply confirmed after infusion in one volunteer of either ozonated blood or the gluco-peroxide solution. However, both types of infusions have already been practiced in hundreds and thousands of patients.

If LOPs remained stable in the circulation during a 20 min infusion, we would have measured their progressive increase in serial plasma samples collected during the infusion. Figure 5A clearly shows that TBARS hardly increased during the infusion of ozonated blood and returned to normal levels during the next 30 min. Infusion of the gluco-peroxide solution did not affect TBARS values either during infusion or thereafter (figure 5B). Likewise, the kinetics of PTG values does not show any modifications. Neither type of infusion caused any acute or chronic side effects.

6) Effect of a treatment cycle of the gluco-peroxide solution in age-related macular degeneration patients.

With the exception of a palliative effect of oral supplementation of antioxidants and zinc, there is no orthodox ophthalmological therapy for the atrophic form of ARMD. Unavoidably this disease more or less rapidly progresses to blindness. However, since 1995, we have found that due to retinal ischaemia combined with retinal pigment epithelium and photoreceptors degeneration, the early and not too advanced stages of this disease can benefit from a cycle of 14-16 treatments in two months (twice weekly) of oxygenated-ozonated autohaemotherapy (AHT). Mean distance visual acuity was significantly improved in ARMD patients whereas no significant improvement was observed in the control (oxygen only) group^{15,16}.

Unfortunately a sizable proportion of women have poor venous accesses which impede the AHT treatment. The gluco-peroxide solution can nonetheless be infused via small veins visible on the back of the hand. So far, in close collaboration with the ophthalmologist, we have performed a full cycle (14 treatments during seven weeks) in six women. The ophthalmologic control performed at the end of the cycle disclosed an increase in visual acuity from 0.15 to 0.23 (an average increase

of 53%) in four women. The improvement was minimal in one woman and none in another probably owing to irreversible photoreceptors degeneration. Considering that there are no other therapeutic options, the improvement in visual acuity, though modest, is highly appreciated by patients because they become more independent and report a better quality of life. The fact that they show an excellent compliance during the successive maintenance therapy is the best demonstration of the therapy's value. No control arm (5% glucose solution only) could be carried out because although treatments are free of charge, it would be unethical. There are no side effects and the majority of patients currently report feeling well during the course of therapy.

Discussion

Today H₂O₂ is acknowledged as one of the crucial ROS and an early and effective ozone messenger. By activating glycolysis, ATP and 2,3-DPG formation in erythrocytes, these biochemical changes lead to improved oxygen delivery in ischaemic tissues^{15,16}. In lymphocytes, the transient increase in H₂O₂ in the cytoplasm activates a tyrosine kinase, which, by phosphorylating IκB, detaches it from the inactive complex NFκB-IκB allowing rapid migration of the heterodimer to the nucleus resulting in the successive synthesis of several proteins¹⁵. Even a minimal activation of platelets enhances the release of growth factors^{34,35} and, on endothelial cells, H₂O₂ determines an increased production of NO¹⁷⁻¹⁹. Thus direct infusion of the gluco-peroxide solution triggers a number of biochemical pathways that can eventually result in therapeutic effects.

From our data (figure 5), it appears that the infusion of this solution barely increases the plasma levels of peroxidation end-products, while this aspect is slightly more evident after the infusion of ozonated blood into the donor. The fact that TBARS and PTG plasma levels remain practically unmodified is due firstly to judicious dosages, secondly to the lower oxidizing property of H₂O₂ in comparison to ozone, thirdly to the prompt reducing activity of blood antioxidants and fourthly to the association of a number of processes (dilution in body fluids, neutralization, enzymatic detoxification and excretion of LOPs) operating simultaneously and effectively *in vivo*. These considerations explain why the use of either ozonated blood or the gluco-peroxide solution is never accompanied by any acute or chronic toxicity. We have calculated that by virtue of the protective processes described, the final circulating levels of LOPs dur-

ing and after reinfusion of ozonated blood remain at submicromolar levels. This result is important³⁶ because the transitory presence of LOPs is likely to be responsible for the upregulation of antioxidant enzymes (SOD, GSH-peroxidase, G-6PD) we demonstrated in erythrocytes *in vivo* during a therapeutic cycle of ozone therapy^{15,16,33,37,38}. This paradoxical result has a great practical validity and is due to repeated, calculated, acute oxidative stresses able to induce a positive response similar to the phenomenon of small, repeated ischaemic heart-conditioning effects.

We emphasize that hydrogen peroxide should never be added to physiological solution (saline) owing to the risk of forming caustic OCl⁻. Moreover we condemn the infusion of ozonated saline in vitro as well as in patients: this practice has become common in Russia and, regrettably, is also used by a few charlatans in Italy without understanding that they can harm the patient by infusing an irritant as powerful and toxic as OCl⁻.

Work in progress aims to compare laboratory and clinical results by testing the classical HAT and the gluco-peroxide solution in vasculopathies, ARMD, infectious and degenerative diseases. This rationale behind this line of work is that H₂O₂ is one of the most important early ozone messengers. However the question whether H₂O₂ is as effective as ozonated blood autotransfusion remains open because late products, like LOPs, appear to be scarcely generated *in vivo* owing to rapid re-

duction of hydrogen peroxide. Needless to add, although gluco-peroxide infusion does not present the risk of direct gas administration, it must be performed slowly to avoid any risk of oxygen embolism and potential venous irritation. *A clear disadvantage to bear in mind is that the gluco-peroxide solution cannot be used in diabetics.* Nonetheless we feel that this approach deserves to be pursued because it has the following advantages: A) ozone generators, with all their problems and cost, would become superfluous. B) The cost of the gluco-peroxide solution is almost negligible: the preparation of the solution is simple and well standardized, it is far more stable than ozone and can be transported and administered anywhere. C) One needs only reagent grade H₂O₂ (30%), a few ampoules of sterile bidistilled water, the 5% glucose solution flask, a few plastic disposable tools and, with good care and a little experience there is no need for electricity or a sterile hood. The advantage is that this therapy could be performed in poor countries in remote corners of our planet to alleviate serious diseases. We should not forget that the majority of the Earth's population receives minimal medical assistance.

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