
INTERNATIONAL JOURNAL OF OZONE THERAPY

formerly RIVISTA ITALIANA DI OSSIGENO-OZONOTERAPIA

THE OFFICIAL JOURNAL OF WFOT - WORLD FEDERATION OF OXYGEN-OZONE THERAPY,
FIO - ITALIAN FEDERATION OF OZONE THERAPY, ACEOOT - SPANISH ASSOCIATION OF OZONE THERAPY,
HELLENIC, INDIAN, SLOVACH AND CHINESE NATIONAL SOCIETIES

Index

■ Editorial			
DECADE REVIEW OF OZONE THERAPY IN CHINA X. He	53	ARE DIALYSIS DEVICES USABLE AS OZONE GAS EXCHANGERS? V. Travagli, I. Zanardi, A. Gabbrielli, E. Paccagnini, V. Bocci	80
■ Original Articles			
ROLE OF OZONE/OXYGEN IN FIBROBLAST GROWTH FACTOR ACTIVATION. DISCOVERING THE FACTS L. Re, G. Martínez-Sánchez, G. Perez-Davison, M. Siritto	55	LUMBAR VERTEBRAL DISC PATHOLOGY. NEW FRONTIERES: OZONE + STEM CELLS L. Brina, A.G. Popandopulo	86
BACTERIAL CULTURE EXPERIMENTS OF MEDICAL OZONE B. Yu, Q-R Lin, B-W Wang, Y-F Zhang, X-F He	59	LUMBAR DISC DISEASE: 10 YEARS OF EXPERIENCE IN PARAVERTEBRAL INFILTRATION WITH OXYGEN-OZONE MIX L. Brina	93
MECHANISM OF ACTION AND CHEMICAL-BIOLOGICAL INTERACTIONS BETWEEN OZONE AND BODY COMPARTMENTS: A CRITICAL APPRAISAL OF THE DIFFERENT ADMINISTRATION ROUTES V. Bocci I. Zanardi, D. Michaeli, V. Travagli	63	MAY OXYGEN-OZONE THERAPY IMPROVES CARDIOVASCULAR DISORDERS? V. Bocci, V. Travagli, I Zanardi	97
THE IRRATIONALITY OF A NON-SPECIFIC IMMUNOMODULATION THERAPY USED IN CARDIOVASCULAR DISEASES DESERVES A CRITICAL COMMENT V. Bocci, I. Zanardi V. Travagli	78	CURRENT CONCEPTS OF OXYGEN OZONE THERAPY FOR DENTISTRY IN THE UNITED STATES J.A. Rothchild, R.E. Harris, P.J. Mollica	105
		OZONE AND OZONATED OILS IN SKIN DISEASES: A REVIEW V. Travagli, I. Zanardi, G. Valacchi, V. Bocci	109
		■ Errata-Corrige Non-Invasive Approaches to Back Pain in Patients with Somatization A. Bariselli	127
		■ Information & Congresses	119, 128
		■ FIO Application Form	132
		■ Instructions to Authors	134

Cover: L. Esposito - *Pulcinella a braccia aperte*
"Welcome to Bologna" - 2003 Bronzo - 220×145×40 cm

DIAGNOSI & RIABILITAZIONE FORMATO FAMIGLIA.

Sì, proprio così.

A Brescia è nato un Centro Polifunzionale in grado di soddisfare i bisogni di tutta la famiglia. Dove il rapporto umano viene prima di tutto. E dove specialisti e fisioterapisti di alto livello si incontrano con metodi, sistemi e tecnologie avanzatissimi.

- Laserterapia
- Correnti Analgesiche
- Massoterapie
- Rieducazione e Riabilitazione
- Ozonoterapia
- Visite Specialistiche
- Tac
- Ecografie
- Ecodoppler
- Tecar
- Spine-Lab



POLIAMBULATORIO OBERDAN / info@poliambulatorioberdan.it
Via G. Oberdan, 140
25128 BRESCIA

Tel. 030 3701312
Fax 030 3387728

NUOVO A BRESCIA

In collaborazione con

Terme di Angolo

www.termediangolo.it

salute · wellness · beauty · fitness



Editorial

Decade Review of Ozone Therapy in China

X. HE

Department of Interventional Therapy, Nanfang Hospital, Southern Medical University; Guangzhou, China

It has been ten years since ozone therapy was first performed in 2000 in China. In the past decade, ozone therapy has made major achievements. It was only used for lumbar disc herniation initially, but now it is used to treat various pains, osteoarthritis, gynecological inflammation, ulcers, viral hepatitis, cerebral infarction and so on. Currently, hundreds of hospitals have already undertaken the program of ozone therapy, and according to incomplete statistics, hundreds of thousands of patients receive this therapy each year. Patients with cervical and lumbar disc herniation number more than 50,000 each year, with a more than 80% excellent outcome.

In China, HBV prevalence in the population is very high, and the efficacy of ozone autohemotherapy is equivalent to that of oral antiviral drugs, thus for patients with drug resistance, ozone autohemotherapy is the best choice. Currently, many hospitals use ozone therapy for cerebral infarction in clinical research, and this has undergone rapid development.

The preliminary study outcomes show that ozone therapy can reduce cerebral edema and promote brain tissue repair, which has been the new topic in the last two years. Ozone therapy for tumors is still in its infancy, but it has seen some good signs. In September 2008, with the strong support of the President of the China Chapter of the World

Medical Association Pain, Prof. JiaXiang Ni, the China Federation of Ozone Therapy (CFOT) was established, with Prof. Xiaofeng He as director, liver specialist Prof. Yabing Guo, orthopedic specialist Prof. Bin Yu and neurological expert Kairun Peng as core strengths of the federation.

In February 2009, a book called "The clinical application of ozone therapy" edited by the Xiaofeng He was officially published. It is the first professional book on ozone therapy in China, collecting the latest researches in various fields. The first and second annual conferences have been successfully held since the establishment of the Federation, with more than 300 participants at each session and 288 members. Around 30 hospitals or medical units have been granted "Demonstration Unit for Ozone Therapy" or "Standardized Unit of Ozone Therapy". Meanwhile, with the financial support of the German HUMARES Company, the "NANFANG HOSPITAL-HUMARES clinical training center of ozone therapy" was established specializing in the systematic training of physicians who are fresh to ozone therapy. Training contents include ozone therapy for disc herniation, liver disease, cerebrovascular disease, osteoarthritis, and animal experimental practice.

We believe that in the near future, ozone therapy will benefit more and more patients like other treatments.

Role of Ozone/Oxygen in Fibroblast Growth Factor Activation. Discovering the Facts

L. RE^{1,2}, G. MARTÍNEZ-SÁNCHEZ², G. PEREZ-DAVISON², M. SIRITO³

¹ Pharmacology, D.I.S.M.A.R., University of Ancona; Ancona, Italy

² Medinat srl Clinic; Camerano, Italy

³ Anti Aging Center, Thermal Institute of Angolo Terme; Angolo Terme, Italy

Key words: ozone, fibroblast growth factor, platelet rich plasma

SUMMARY - Basic fibroblast growth factor (bFGF) is a pleiotropic mitogen which plays an important role in cell growth, differentiation, migration and survival in different cells and organ systems. Application of bFGF has been shown to promote cellular proliferation and collagen synthesis in vivo. FGF is markedly up-regulated following bone or tendon injury and active at multiple stages of the healing stimulation process, local blood circulation, lipolysis and smooth muscle. Looking at the physical and chemical properties of the ozone molecule, the present work deals with its possible therapeutic action as an FGF activator. Incubation (2 h) of platelet-rich plasma with 80 µg/mL O₂/O₃ increases the basal concentration of FGF by approximately 600%. This fact in combination with previous demonstration of the stimulating action of O₃, releasing other platelet factors may potentially allow autologous treatment in aesthetic and clinical tissues conditions in which FGF has a leading role. The versatility and broad beneficial effect of ozone has become evident in orthopedics, cutaneous and mucosal infections as well as in dentistry. The induction of FGF and other growth factors by ozone can support and potentiate those applications.

Introduction

Application of basic fibroblast growth factor (bFGF) has been shown to promote cellular proliferation and collagen synthesis in vivo. In vitro and in vivo studies have shown that bFGF is both a powerful stimulator of angiogenesis and a regulator of cellular migration and proliferation, and is markedly up-regulated following tendon injury and active at multiple stages of the healing process¹. Use of FGF as a therapeutic agent for the treatment of ischemic cardiovascular disease is promising and clinical trials are in progress. FGF has pleiotropic roles in many cell types and tissues: it is a mitogenic, angiogenic and survival factor involved in cell migration, cell differentiation and in a variety of developmental processes. Although devoid of signal peptide, FGF could be secreted. It acts mainly through a paracrine/autocrine mechanism involving high affinity transmembrane receptors and heparin sulphate proteoglycan low affinity receptors, but also through a still unknown intracrine process(es) on intracellular targets. FGF has many biological functions which are probably isoform-specific. bFGF may sensitively regulate local bone resorption and remodeling through direct and indirect mechanisms that promote angiogenesis and osteocyte recruitment, formation, differen-

tiation and activated bone pit resorption². It has been demonstrated that ozone (O₃) can promote platelet aggregation particularly when heparin is used as an anticoagulant. Platelet-rich plasma (PRP) treated with O₃ significantly increases the amount of platelet-derived growth factor (PDGF), transforming growth factor b1 (TGF-b 1) and interleukin-8 (IL-8). These factors are released in a dose-dependent manner after ozonation of heparinised PRP samples. These findings may partially explain the enhanced healing of topical ulcers in patients with chronic limb ischemia treated with O₃ autohaemotherapy (O₃-AHT)³. FGF plays a role in various stages of development and morphogenesis, as well as in angiogenesis and wound healing processes. That is why the aim of this study was to evaluate the efficacy of O₃ to induce the release of FGF in PRP.

Materials and Methods

The O₂/O₃ mixture was generated just before application by an Alnitec Ozo2Futura device (Italy). Ozone obtained from medical grade oxygen represented about 0.4-0.5% of the gas mixture. The ozone concentration was measured using a built-in UV spectrophotometer at 254 nm.

Reagents

Anticoagulants were either heparin (calcium salt, 30 IU/mL blood) normally used for therapeutic purposes (Clarisco, Teofarma srl, Pavia, Italy) or ACD (citric acid, sodium citrate, glucose) (Haemonetics, Braintree, MA, USA).

Preparation of platelet-rich plasma samples

Serum, ACD or heparinised PRP were prepared from the same blood samples (18 mL) drawn from five fasting (12h) non-smoker volunteers between the ages of 23 and 60 years, who were considered to be healthy and had not ingested platelet-active medication for at least two weeks. This study was approved by an institutional review board (Scientific and Ethics Committees of the Institution) in accordance with the principle of the Declaration of Helsinki concerning the Ethical Principles for Medical Research Involving Human Subjects⁴. All volunteers signed an informed consent form before being enrolled. All patients were given adequate information (characteristics of the study, benefits and possible side-effects). Before enrolling, all participants attended a training programme to familiarize with the study objectives.

Nine parts blood were mixed with one part of saline or anticoagulated with either one part ACD or with one part of saline containing heparin so the final concentration was 30 IU/mL. Blood was centrifuged at 200 g for 20 min and platelets were measured with a Coulter counter. An average platelet count of 3×10^8 /mL serum / plasma was used.

Treatment of biological samples

A volume of 0.4 mL of PRP was mixed with 25 μ L saline, 25 μ L of CaCl 10%, 25 μ L of saline plus 0.4 mL of O₂/O₃ gas mixture (O₃ concentration of 80 μ g/mL) or 25 μ L of saline plus 0.4 mL of O₂. O₂/O₃ or O₂-treated samples were collected with a silicone coated disposable syringe and immediately introduced into a second syringe containing an equivalent volume of PRP via a 'y' connector. Final gas pressure remained at normal atmospheric pressure. The PRP samples were gently but continuously mixed with the gas for up to 30 s and afterwards the "y" were dispensed into test tubes for FGF analysis. After 2h incubation each sample was immediately centrifuged at 10 000 g for 20 min at 2°C and the supernatant platelet-free was frozen at -70°C until determination of FGF.

Immunoassay

Immunoassays of human bFGF were carried out using immunoassay kits produced by EMELCA Bioscience (Breda, Netherlands). On the basis of preliminary tests samples were diluted. A Bio-Rad

680 microplate reader (Hemel Hempstead, UK.) at 450 nm was used to read the samples absorbance. Samples were tested at least in duplicate against the appropriate standards.

Statistical analysis

The Outliers preliminary test for detection of error values was initially applied. Afterwards, data were analyzed for normality using the Shapiro-Wilk W test followed by homogeneity variance test (Levene). In addition, descriptive statistics was done. Results are expressed as the mean \pm the standard deviation of the mean (S.D.). A software package was used for data collection and statistical analysis (Statistics for Windows 17.0.2, USA). The significance of the differences between the means in each group was analyzed by one-way analysis of variance (ANOVA). The level of statistical significance was set at $p < 0.05$ for both inter and intra-groups analysis.

Results

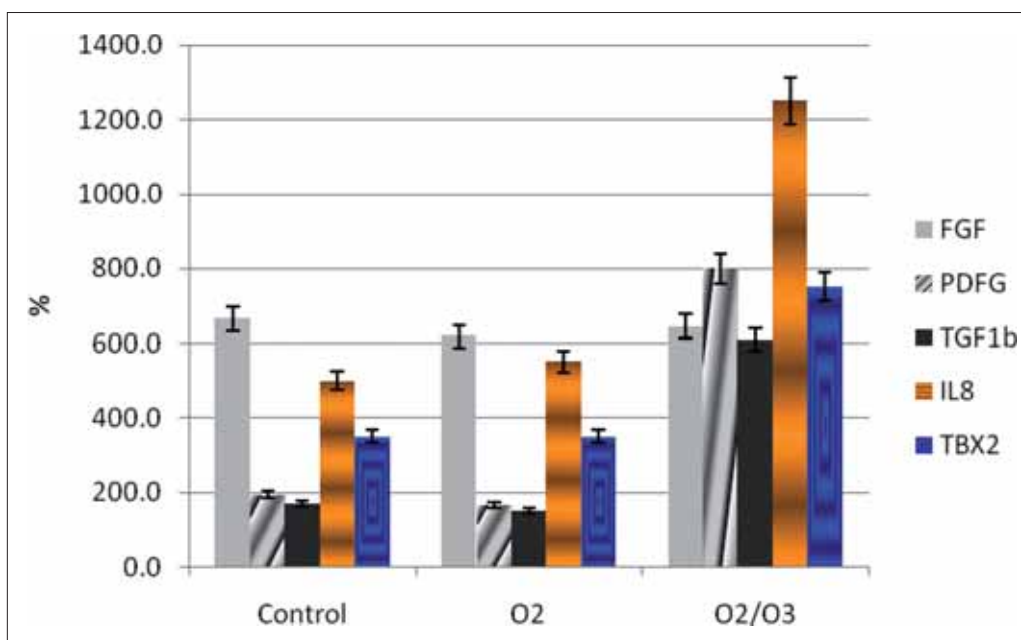
The concentration of FGF was increased by approximately 580-700 percent after 2h incubation of PRP with saline, Ca²⁺, O₂ or O₂/O₃ compared to the basal concentration 1.636 ± 0.969 pg/mL. Statistical differences were not detected among the concentrations of FGF from platelets collected in ACD, heparin or those obtained from serum. The intra group analysis (different treatment between the same samples) showed no significant differences in FGF concentration in samples of PRP treated with saline, Ca²⁺, O₂ or O₂/O₃ (Table 1).

Discussion

It has been demonstrated that exposure of PRP to O₂/O₃ stimulates the release of different growth factors^{3,5}, but the effect of this procedure on the induction of FGF has not been reported. As demonstrated (Table 1) during O₂/O₃ exposure, PRP released similar quantities of FGF as when incubated with other substrates. FGF has an essential role in the differentiation of stem cells^{6,7} and is a potent in vitro mitogen for capillary endothelial cells, stimulating angiogenesis in vivo, and may participate in tissue repair². In contrast with other PRP treatments O₂/O₃ exposure releases significant ($p < 0.05$) quantities of other platelet growth factors (Figure 1), and this pool of factors is thought to participate in the regeneration of tissues.

PRP is a rich source of growth factors and promoted significant changes in monocyte-mediated

Figure 1 Shows that incubation of PRP collected in heparin, untreated (control) or exposed to O_2 or O_2/O_3 dramatically increased the release of platelet growth factors compared to the basal level. The highest and significant ($p < 0.05$) increment was reached in the O_2/O_3 treated group for PDGF, TGF1b, IL8 and TBX2. However, the increment in the concentration of FGF has the same proportion in groups untreated or exposed to O_2 or O_2/O_3 samples.



proinflammatory cytokine/chemokine release. LXA4 was increased in PRP, suggesting that PRP may suppress cytokine release, limit inflammation, and, thereby, promote tissue regeneration⁸. Platelet activation allows access to autologous growth factors which by definition are neither toxic nor immunogenic and capable of accelerating the normal processes of bone regeneration. PRP can thus be considered a useful instrument for increasing the quality of regenerated bone⁹, wound healing¹⁰, healing of injury-associated soft tissue defects¹¹, chronic non-healing tendon injuries including lateral epicondylitis and plantar fasciitis and cartilage degeneration¹².

Ozone therapy has been used for many years as a method ancillary to basic treatment, espe-

cially in those cases in which traditional treatment methods do not give satisfactory results, e.g. skin loss in non-healing wounds, ulcers, pressure sores, and fistulae. Clinical results demonstrate the efficacy of this procedure in post-surgical and post-trauma complications¹³, diabetic food¹⁴, and others. Some recent studies also demonstrate that part of the wound repair mechanisms of ozone or ozone-derived products are associated with an increased expression of PDGF, TGF-beta, and VEGF¹⁵.

As demonstrated, ozone does not modify the increment in the release of FGF from PRP. However, O_2/O_3 stimulates the release of a number of growth factors present in PRP, including PDGF, TGF-b1, IL-8 and TBX2. The ability of ozone to

Table 1 Release of bFGF from human platelets during 2h incubation. The PRP samples in saline, heparin or ACD were not exposed (control), or exposed to Ca^{2+} , O_2 or O_2/O_3 (80 $\mu\text{g}/\text{mL}$) for 30 s before incubation.

Sample		NaCl	O_2/O_3 80 $\mu\text{g}/\text{mL}$	O_2	Ca^{2+}
Plasma collected in heparin	Mean	10.84	10.50	10.07	11.21
	S.D.	1.49	1.40	1.26	1.89
	% I	662	642	616	685
Plasma collected in ACD	Mean	11.61	9.83	9.62	10.61
	S.D.	3.32	2.37	2.33	2.83
	% I	709	601	588	648
Serum	Mean	9.86	9.50	9.58	10.96
	S.D.	2.61	1.43	2.41	2.89
	% I	602	580	586	670

stimulate the release of other important growth factors from platelets, like vascular endothelial growth factor (VEGF) and insulin-like growth factor (IGF), should be tested. In the early stages of wound healing, platelets are activated by thrombin and collagen, and growth factors are released by these activated platelets to facilitate repair and healing. The sequence of events leading to bone formation and wound healing (chemotaxis, cell migration, proliferation, and differentiation)¹⁶ are regulated by growth factors¹⁷, many of which are present in PRP. For example, the recruitment of mesenchymal stem cells and progenitor cells to the site of bone regeneration is mediated by collagen as well as chemotactic factors such as PDGF and TGF- β 1. Moreover, PDGF and TGF- β 1 stimulate cell proliferation¹⁸, and TGF- β 1 also induces the osteogenic differentiation of mesenchymal stem cells¹⁹. Osteoblast differentiation is controlled by IGFs and bone morphogenetic proteins¹⁶, and VEGF is critical in stimulating the angiogenesis necessary for bone formation and remodeling.

Conclusions

The compelling advantages of PRP for bone repair and wound healing and the capacity of ozone to stimulate the release of growth factors is an important point for future research on this topic. Future studies will also focus on the optimization of release of growth factors. The objective is to tailor the release cascades to match those required for tissue regeneration, enabling essential factors to be available at physiologically effective concentrations and during critical periods of remodelling.

Basal concentration of bFGF in non-treated serum/plasma was 1.636 ± 0.969 pg/mL. The mean platelet concentration used was $447.8 \cdot 10^3/\mu\text{L}$. ACD, citric acid, sodium citrate, glucose. PPR, platelet-rich plasma. I% increment in bFGF compared to basal bFGF concentration. Non-statistical differences were detected when compared to the concentration of FGF among groups. Statistical differences ($p < 0.001$) were detected between basal concentration and post incubation samples in all cases.

References

- 1 Devescovi V, Leonardi E, Ciapetti G, et al. Growth factors in bone repair. *Chir Organi Mov*. 2008 Dec; 92(3): 161-168.
- 2 Zakrzewska M, Marcinkowska E, Wiedlocha A. FGF-1: from biology through engineering to potential medical applications. *Crit Rev Clin Lab Sci*. 2008; 45(1): 91-135.
- 3 Bocci V, Valacchi G, Rossi R, et al. Studies on the biological effects of ozone: 9. Effects of ozone on human platelets. *Platelets*. 1999; 10(2-3): 110-116.
- 4 WMA. World Medical Association Declaration of Helsinki. Ethical Principles for Medical Research Involving Human Subjects. Volume 15. Adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964: *J Int Bioethique*. 2004; 124-129.
- 5 Valacchi G, Bocci V. Studies on the biological effects of ozone: 10. Release of factors from ozonated human platelets. *Mediators Inflamm*. 1999; 8(4-5): 205-209.
- 6 Lavaute TM, Yoo YD, Pankratz MT, et al. Regulation of neural specification from human embryonic stem cells by BMP and FGF. *Stem Cells*. 2009; 27: 1741-1749.
- 7 Eiselleova L, Matulka K, Kriz V, et al. A complex role for FGF-2 in self-renewal, survival, and adhesion of human embryonic stem cells. *Stem Cells*. 2009; 27(8): 1847-1857.
- 8 El-Sharkawy H, Kantarci A, Deady J, et al. Platelet-rich plasma: growth factors and pro- and anti-inflammatory properties. *J Periodontol*. 2007 Apr; 78(4): 661-669.
- 9 Pacifici L, Casella F, Maggiore C. [Platelet rich plasma (PRP): potentialities and techniques of extraction]. *Minerva Stomatol*. 2002 Jul-Aug; 51(7-8): 341-350.
- 10 Gao F, Wang JX, Han Y. [Research advance on application of platelet-rich plasma in wound repair-Review.]. *Zhongguo Shi Yan Xue Ye Xue Za Zhi*. 2009 Jun; 17(3): 840-843.
- 11 Cieslik-Bielecka A, Bielecki T, Gazdzik TS, et al. Autologous platelets and leukocytes can improve healing of infected high-energy soft tissue injury. *Transfus Apher Sci*. 2009; 41(1): 9-12.
- 12 Sampson S, Gerhardt M, Mandelbaum B. Platelet rich plasma injection grafts for musculoskeletal injuries: a review. *Curr Rev Musculoskelet Med*. 2008 Dec; 1(3-4): 165-174.
- 13 Bialoszewski D, Kowalewski M. Superficially, longer, intermittent ozone therapy in the treatment of the chronic, infected wounds. *Ortop Traumatol Rehabil*. 2003 Oct 30; 5(5): 652-658.
- 14 Martinez-Sanchez G, Al-Dalain SM, Menendez S, et al. Therapeutic efficacy of ozone in patients with diabetic foot. *Eur J Pharmacol*. 2005 Oct 31; 523(1-3): 151-161.
- 15 Kim HS, Noh SU, Han YW, et al. Therapeutic effects of topical application of ozone on acute cutaneous wound healing. *J Korean Med Sci*. 2009 Jun; 24(3): 368-374.
- 16 Lu HH, Vo JM, Chin HS, et al. Controlled delivery of platelet-rich plasma-derived growth factors for bone formation. *J Biomed Mater Res A*. 2008 Sep 15; 86(4): 1128-1136.
- 17 Dimitriou R, Tsiridis E, Giannoudis PV. Current concepts of molecular aspects of bone healing. *Injury*. 2005 Dec; 36(12): 1392-1404.
- 18 Canalis E, Pash J, Varghese S. Skeletal growth factors. *Crit Rev Eukaryot Gene Expr*. 1993; 3(3): 155-166.
- 19 Bonewald LF, Mundy GR. Role of transforming growth factor beta in bone remodeling: a review. *Connect Tissue Res*. 1989; 23(2-3): 201-208.

Prof. Lamberto Re
Pharmacology, D.I.S.M.A.R.
University of Ancona
Via Breccia Bianche
60128 Ancona, Italy.
Tel.: +39 071731076, Fax: +39 071731347
E-mail: lambertore@univpm.it.

Bacterial Culture Experiments of Medical Ozone

B. YU¹, Q-R LIN¹, B-W WANG¹, Y-F ZHANG², X-F HE²

¹ Department of Orthopaedics and Trauma, Nanfang Hospital, Southern Medical University; GuangZhou, Guangdong, China

² Department of Interventional Therapy, Nanfang Hospital, Southern Medical University; GuangZhou, Guangdong, China

Key words: medical ozone, bacterial culture

SUMMARY - Medical ozone therapy has been widely used all over the world, but there are few fundamental studies on whether medical ozone includes bacteria and whether it retains its antibiosis ability in vivo. This study is a bacterial culture experiment of medical ozone at different concentrations (10 µg/ml, 30 µg/ml, 50 µg/ml, 80 µg/ml, using or not a disposable gas filter needle when medical ozone is obtained) from a generator 20 cm upper puff towards culture capsules for 20 minutes in a superclean bench. Sixteen Wistar rats were randomly divided into four groups (n=4). Medical ozone at different concentrations (10 µg/ml, 30 µg/ml, 50 µg/ml, 80 µg/ml) was injected into both knee cavities (0.5ml in each knee cavity) of the rats in the four groups, respectively. A disposable gas filter needle was used when medical ozone was injected into the right side, while it was not used for the left sides for control. Twenty-four hours after injection, douche fluid of the joints was obtained for bacterial culture. All blood agar plates for medical ozone gas directly cultured had no colony. As for the douche fluid culture of the joints, the control group and the fourth experimental group (80 µg/ml) had no colony. The other three groups had one blood agar plate with colonies; and the number of colonies fell as medical ozone concentration increased. Therefore, medical ozone may contain bacteria, and the disinfection of medical ozone after injection may weaken or even disappear, thus in the application of medical ozone therapy a disposable gas filter should be used with the medical ozone generator.

Introduction

Medical ozone has a strong oxidizing and bactericidal action in vitro, but its bactericidal action is based on a certain concentration and certain time, because the totally enclosed sterile components of the ozone generator and connecting passageways cannot be ensured. Does medical ozone contain bacteria and still maintain its strong bactericidal capacity after injection and coactions with the anti-oxidation system? This study preliminarily explored these questions.

Materials and Method

An E80 ozone generator (OZONLINE, Italy) was used with adjustable medical ozone level range of 1~80 µmol/ml; pore size 0.22 µm disposable gas filter needle (Dept. of Interventional Therapy, Nanfang Hospital); 16 Wistar rats (Southern Medical University Laboratory Animal Center, weighing 180~220g, no limit to the sex); 32 one-time blood agar culture capsules (Zhengzhou Branch Antu Green Bio-engineering Co., Ltd.);

CO₂ incubator (Queue, USA); superclean bench (Laboratory of Orthopedic Trauma, Nanfang Hospital).

Culture of synovial fluid after intra-articular injection of medical ozone

Sixteen Wistar rats were randomly divided into four groups (n = 4), and 0.5 ml medical ozone at a concentration of 10 µg/ml, 30 µg/ml, 50 µg/ml, 80 µg/ml was injected into double rat knee joint cavities in the four groups, respectively, with the right knee as the experimental side without using a disposable needle filter when obtaining medical ozone and the left knee joint as control side using disposable needle filters.

At first, the rates were fixed in a supine position and intraperitoneal injection of 3% sodium pentobarbital 30 mg/kg was performed for anesthesia. Then, 0.5 ml medical ozone was obtained with a 1ml sterile syringe. When puncturing the lateral knee joint space, the needle may have a clear sense of frustration. If there is no blood when pumping back, it means the syringe needle did not puncture

blood vessels, then medical ozone can be injected into the knee joint cavity of the rats. Medial and lateral knee joint spaces were bulging in the injection process, but there was no swelling in the upper leg or leg, thus we could confirm that the medical ozone was injected into the joint cavity rather than outside the joint. In order to avoiding changes in medical ozone traits, the whole process must be completed within 15s.

24 hours later after medical ozone injection, the bilateral knee joints and the surrounding skin were shaved, and iodine and alcohol were used for skin sterilization. When the rat knee joint flexion was about 80~90°, a slight depression could be seen outside the skin, which is the surface projection of the lateral knee joint space. 0.5 ml sterile PBS solution was injected into the joint cavity for articular cavity perfusion, and then a sterile EP tube was used for collecting the perfusion fluid.

Both kinds of perfusion fluid (experimental side and control side) of the four groups (10 µg/ml, 30 µg/ml, 50 µg/ml, 80 µg/ml) were inoculated onto blood agar Petri dishes on a superclean bench, and those dishes were placed in a CO₂ incubator (culture conditions as 36.5°C, 0.50% CO₂ concentration) for culture. 48 hours later, the situation of each Petri dish colony was observed.

Direct culture in superclean bench

Control group: using a disposable gas filter needle when medical ozone is obtained a sterile hose connected to an ozonizer using a disposable gas filter needle, and openings in the superclean bench,

Table 1 Joint fluid bacteria culture situation*.

Experimental group	Control group		Total
	+	-	
+	0	3	3
-	0	13	13
Total	0	16	16

* "+": indicated that there was bacterial growth; "-" indicated no bacterial growth

Table 2 Experimental group the number of subgroup cases colony.

Group	Colonies number
Medical ozone 10R	13
Medical ozone 30R	8
Medical ozone 50R	2
Medical ozone 80R	0

20 cm of two blood agar plates, open ozonizer 5 min when the concentration stability in 10µg/ml, open the plates. Twenty minutes later placed a CO₂ incubator (culture conditions as 36.5°C, CO₂ concentration of 0.50%), the same method was applied for concentrations of 30 µg/ml, 50 µg/ml, 80 µg/ml of medical ozone respectively.

Experimental group: medical ozone was obtained without using a disposable gas filter needle. A sterile hose connected to an ozonizer without using a disposable gas filter needle, and openings in the superclean bench, 20 cm of two blood agar plates, open ozonizer 5 min when the concentration stability is 10 µg/ml, open the plates, 20 min later placed a CO₂ incubator (culture conditions as 36.5 °C, CO₂ concentration of 0.50%), the same method was applied for concentrations of 30 µg/ml, 50 µg/ml, 80 µg/ml of medical ozone respectively.

Statistical analysis:

SPSS13.0 software was used to analyze the results, and the standard of $\alpha = 0.05$. $P \leq 0.05$ indicated statistically significant difference.

Results

No colonies appeared in the joint cavity douche culture of one experimental group (80 µg/ml medical ozone) and all control groups. Meanwhile, the other three experimental groups (10 µg/ml, 30 µg/ml and 50 µg/ml) had blood agar Petri dish colonies with the number as 13, 8 and 2, respectively.

Specific colony growth is summarized in Tables 1 and 2. There were three petri dishes of bacteria growth in total, all in the experimental groups (10 µg/ml, 30 µg/ml, and 50 µg/ml). The experimental data are not in line with the matching data chi-square test, so only the results could be described. When the medical ozone concentration decreased, the number of colonies in the experimental groups increased significantly.

Discussion

In 1906, in the French city of Nice, medical ozone was used to disinfect water. Then, the sterilization effect of medical ozone was used for disinfection of municipal water by certain countries in the world. Since medical ozone is a strong oxidizer, its sterilization is based on oxidization. And as a broad-spectrum microbicide, its disinfection is 300~600 times faster than chlorine. However,

the disinfection ability of medical ozone is mainly affected by temperature, contact time and medical ozone concentration¹.

Common ozone uses air as a raw material for preparation, containing many nitrogen oxides, thus the concentration cannot be precisely controlled. In this study, the medical ozone was made from pure oxygen by sophisticated medical regulating the medical ozone concentration of the ozone generator, and the medical ozone is a mixture of medical ozone and oxygen, without any other compound. It has many biological effects, such as promotion of glycolysis, regulation of the immune system, alleviating pain, anti-inflammatory, effects of blood rheology, anti-bacteria, anti-mycetes, anti-viruses, and so on. The application of the dosage and the concentration is very important: the medical ozone at too low a concentration is not able to perform its biological effects, and at too high a concentration, especially over the capacity of body's antioxidant enzymes and glutathione, the generated superoxide anion and hyperoxide may cause decomposition of the cell membrane. Prof. Bocci's experiments showed that medical ozone at the concentration of 1~80 $\mu\text{mol/ml}$ had no significant detrimental effect on red blood cells. Therefore, the general application of medical ozone concentrations should be less than 80 $\mu\text{mol/ml}$, usually between 20 ~ 50 $\mu\text{mol/ml}$ ².

In recent years, the sphere of medical ozone therapy has gradually widened. In addition to medical ozone autohaemotherapy and percutaneous treatment of disc herniation, medical ozone has also been widely used in a variety of pain treatments, such as nerve root pain and sciatica, joint peri-arthritis and tenosynovitis. Those therapies have had good effects³⁻⁵. Medical ozone therapy in the treatment of soft tissue pain, such as around the neuralgia, fibromyalgia, joint peri-arthritis, tenosynovitis and tendon, etc. showed its benefits of being inexpensive, efficient and convenient⁶. Many medical ozone therapists announced the superiority of medical ozone and believed that medical ozone itself referred to disinfection, so the risk of infection was extremely low. However, components of the ozone generator do not guarantee sterility, and also for other aspects related to the ozone generator, such as pure oxygen, each port and connecting pipe, pathogens may carry a risk of infection.

Medical ozone disinfection must rely on the concentration as well as a certain time¹, when medical ozone's concentration was below the bactericidal concentration, or the time domain was shorter than a certain period of time, especially when the medical ozone was injected into the body too quickly and coacts with the antioxidant

system in vivo, losing its strong oxidizing characteristics, it may not play a role of sterilization, and may not even kill the pathogens emerging when medical ozone was generated, finally resulting in infection. Serious infections caused by medical ozone therapy were also occasionally reported, for example, Gazzeri et al.⁷ reported medical ozone percutaneous treatment of patients with disc herniation: three days after treatment medical ozone-related sepsis broke out, and the cause might be the weak concept of aseptic technique, but infection caused by medical ozone pollution could not be ruled out.

The problem is the concern of medical ozone therapists and patients, having great relation to patients' acceptance and confidence in medical ozone therapy, and certainly being related to the application and promotion of medical ozone therapy. Therefore, we carried out a preliminary test, trying to provide the relevant experimental data.

In the first part of the experiment, with or without filter needles, each concentration of medical ozone exposure of dishes was cultured for 20 minutes. After 24-hour culture, there was no bacterial growth. Two reasons may account for this: \square no concentrations of oxygen-ozone mixture included bacteria; \square a certain period of time later, the bacteria was disinfected by the mixture. In the second part, three experimental groups showed positive results, with bacterial growth inside the inoculating loop's scarification, which meant possible pollution in the period of culture, indicating bacteria in the joint fluid. The source of the bacteria might be as follows: \square pollution during operation; \square medical ozone contains bacteria. Although experiments were done in SPF-class laboratory or in a super-clean bench, we still could not guarantee the total enclosed sterility of all operations. However, there was no bacterial growth in the groups subjected to disposable filter needles, which prompted that the application of filter needle may be a great factor to reduce pollution. When medical ozone was polluted by bacteria and other pathogens, or pathogens within itself, and when the amount was small, its concentration was restricted, or might not kill the bacteria it contains, raising the possibility of infection.

The number of colonies increased as medical ozone concentration reduced, indicating even the inhibitory capacity of a high concentration of medical ozone might still be stronger than a low one. As the sample was limited, we could not come to the conclusion that when medical ozone concentration increases, its ability to disinfect the body is enhanced and it is also indefinite whether the concentration is up to 80 $\mu\text{mol/ml}$, the infection could be avoided.

Conclusion

Direct bacteriological testing of medical ozone failed to detect bacteria, but when medical ozone was injected into the articular cavity, the results of joint fluid culture were partially positive, suggest-

ing that there may be bacteria in medical ozone and in vivo medical ozone may not perform its powerful bactericidal action. Direct injection of medical ozone carries the risk of infection. In the use of medical ozone, we suggest using filtering equipment to prevent pollution and infection.

References

- 1 Lin A-H, Rao J, Qin Y-M, et al. Bactericidal effects of medical ozone due to cable analysis. *Hubei J Preven Med.* 2002; 13 (6): 7-8.
- 2 Bocci V. *Medical ozone - a new medical drug.* Netherlands: Springer; 2005.
- 3 Wen R-H, Wang J-H, Du H-J. Medical ozone treatment of 58 cases of fibromyalgia syndrome. *China Rehabilitation.* 2007; 1: 72.
- 4 Biao K, Wu X-M, Chen Y-F, et al. Medical treatment of peri-arthritis of shoulder injection of medical ozone. *Intervent Imag Treat Chinese Studies.* 2006; 5: 341-343.
- 5 He Q, Xu P-X, Du F. Common sports department of soft tissue injury pain treatment O_2-O_3 clinical observation. *J Neck Pain.*, 2006; 2: 133-134.
- 6 He X-F, Li Y-H, et al. Medical ozone treatment of lumbar disc herniation clinical analysis of 600 cases. *Intervent Imag Treat Chinese Studies.* 2005; 2: 338-341.
- 7 Gazzeri R, Galarza M, Neroni M, et al. Fulminating septicemia secondary to oxygen-medical ozone therapy for lumbar disc herniation: case report. *Spine.* 2007; 32 (3): 121-123.

He Xiaofeng MD, PhD
Department of Interventional Therapy
Nanfeng Hospital, Southern Medical
University
No. 1838 North Guangzhou Avenue
Guangzhou
510515 China
E-mail: ozonetherapy@126.com

Mechanisms of Action and Chemical-Biological Interactions Between Ozone and Body Compartments: A Critical Appraisal of the Different Administration Routes

Velio Bocci^{1,*}, Iacopo Zanardi², David Michaeli³ and Valter Travagli²

¹Department of Physiology, ²Department of Pharmaceutical Chemistry and Technology, University of Siena, Siena, Italy, ³Department of Neurosurgery, Rabin Medical Center, Tel-Aviv University Medical School & Microdel Idea Center LTD, Israel

Abstract: After a long initial stage obscured by empirism and misconceptions, oxygen-ozonotherapy has now become a scientific discipline where the reactions between ozone and human blood are within the realm of orthodox biochemistry, physiology and pharmacology. Most of the basic mechanisms of action have been clarified and ozone can be considered a pro-drug, which almost instantaneously reacts with antioxidants and unsaturated fatty acids. These reactions generate the actual ozone messengers represented by either hydrogen peroxide as a fast acting compound or a variety of lipid oxidation products as late effectors. While ozone is totally consumed, micromolar amounts of these messengers are able to enhance the delivery of oxygen via erythrocyte activation, the immune system by a bland leukocyte stimulation and most of the remaining body cells by up-regulating the antioxidant system. The hazard of ozone toxicity has been dispelled by using the gas only within a dose range perfectly calibrated against the potent blood antioxidant capability. Ozonotherapy can be very useful in patients with chronic vascular disorders and ischemic problems and should be extensively used by official medicine. An extraordinary facet of ozone is its medical application versatility, as represented by several administration routes, and the minimal cost of this drug.

Key Words: Ozonotherapy, oxidative stress, antioxidants, ozone tolerance, hormesis, vascular diseases.

INTRODUCTION

Ozonotherapy is almost a century old as it was firstly used as a potent disinfectant for treating gaseous gangrene in German soldiers during World War I. At about the same time, Stoker [1] reported the ozone treatment of several medical cases. It was unfortunate that Christian Friedrich Schönbein, who discovered ozone in 1840, could not take advantage of ozone when he contracted and died from a *Bacillus anthracis* infection in 1868. A leap forward was made by the physicist Joachim Hänsler when, in the 70s, invented the first medical ozone generator thus allowing the possibility of using ozone in medicine. Hans Wolff [2] deserves the credit for having developed the methodology of the ozonated autohemotherapy by exposing human blood in a disposable, ozone-resistant glass bottle to a volume of gas composed by a mixture of medical oxygen (about 95%) and the extemporaneously generated ozone (about 5%). However, only at the end of the century, modern ozone generators, including a UV photometer (252.6 nm) measuring in real-time the ozone concentration, became available and permitted a real progress. In 1995 the National Institutes of Health (Bethesda MD, USA) included ozone and hydrogen peroxide therapy among the pharmacological and biological treatments as alternative and complementary therapies [3]. In spite of this, only about fourteen States of the USA permit to practice ozonotherapy while the Food and Drug Administration continues to deny permission to use ozone in medicine. The

FDA is slow in adjourning its decision because ozone has been badly used in the 90s by directly injecting the gas mixture intravenously into HIV patients, thus procuring deadly oxygen embolism. However, in the patients' interest, it is time that the FDA revises its negative position.

During the last decade a number of scientific studies [4-6], reviews [7-9] and books [10,11] have allowed to clarify that ozone dissolves in the water of plasma very rapidly and switches on a number of chemical reactions, which, on one hand, lead to its exhaustion, while, on the other hand, generate new chemical compounds able to trigger a number of biochemical reactions. Thus now ozone is considered a pro-drug implying that pharmacological or eventual side effects are due to its generated messengers. At variance with other complementary approaches, where the mechanisms of action remain hypothetical or scientifically not verifiable, oxygen-ozonotherapy, after some initial difficulties due to misconceptions and empirism, has reached a stage where the reactions elicited by ozone in human blood and other biological fluids are within the realm of orthodox biochemistry, physiology and pharmacology. This is so true that ozone, in itself a strong and dangerous oxidant, can be used as a real medical drug provided that both its precise concentrations (hence the dose equal to the product of the precise ozone concentration with the gas volume) and the antioxidant capacity of the biological substrates are known. The actual understanding of the various biochemical reactions, the chemical characteristics of the generated messengers, their interaction with physiological components and their pharmacodynamic allow to recommend oxygen-ozonotherapy in vascular disorders characterized by ischemia and chronic oxidative stress where

*Address correspondence to this author at the Department of Physiology, University of Siena, Via Aldo Moro 2, 53100 Siena, Italy; Tel: +39 (0) 577 234226; Fax: +39 (0) 577 234219; E-mail: bocci@unisi.it

orthodox medication, although useful, still present limitations [8,11].

The main aim of this review is to critically analyze all the involved compounds, their biological activities and potential toxicity for justifying the use of ozone by different administration routes in some pathologies. It has become obvious that ozonotherapy cannot be explained by a straightforward interaction between a molecule and its receptor because of the number of messengers and of their different fate and lifetime in the organism. This complexity is not a disadvantage and it seems to result in the synergism of different biological activities. Therefore ozonotherapy is now considered a modifier of the biological response, that is needed in chronic pathologies usually complicated by a chronic oxidative stress. Moreover it must be emphasized that ozonotherapy represents another example of stimulatory responses following a precise stimulus below the toxicological threshold. The concept of "hormesis" has been masterly exemplified by Calabrese [12] and, interestingly, the ability of some medical gases (NO, CO, H₂, H₂S, Xe and O₃) used at very low concentrations to ameliorate oxidative stress has been further stressed by Nakao *et al.* [13].

THE DOGMA OF OZONE TOXICITY IS WELL DISPROVED IN THE CASE OF OZONETHERAPY

Ozone is a very reactive gas and it has an oxidation potential of 2.07 Volts. In the stratosphere the ozone layer with an average concentration of 10 ppm (0.01 µg/ml) is very useful for avoiding an excessive UV solar radiation on living

beings. Chemists, lacking an ample knowledge of the biological system, are concerned about its medical use and tend to create a diffused scepticism. Moreover, ozone toxicity for the pulmonary system during prolonged inhalation of photochemical smog containing 0.2 ppm (0.0002 µg/ml) ozone is well known. Any possible leakage of ozone must be avoided and not only children, asthmatics, smokers and elderly people but also normal adults can be adversely affected [14,15]. Chronic oxidative stress induced by ozone in the lungs causes a steady release of a huge amount of peroxidative products and proinflammatory cytokines which, by easily overwhelming the antioxidant defences present in the very thin film of surfactant (about 0.1 µm), layered over as many as 70 m² of alveoli, not only damage the respiratory surface but, after entering the circulation, cause chronic inflammations in several organs [16].

How then ozone can be medically useful? Luckily, after about 2.5 billion years of terrestrial life in oxygenated air, a very potent and multiform antioxidant system (Table 1) has developed in biological fluids and cells and it has become almost completely protective.

A comparative evaluation of the antioxidant system in the human lungs and blood has been very instructive [17]: the enormous respiratory surface is covered with only about 20-40 ml of endothelial lining fluid (ELF) containing a modest amount of hydrophilic antioxidants, and less than 100 mg of albumin to neutralize the ozone insult. On the other hand, about 2.8L of plasma and a further 10-12 L of interstitial fluids contain as many as about 470 g of albumin, which is

Table 1. The Antioxidant Nonenzymatic and Enzymatic Systems in Biological Fluids

NONENZYMATIC	
Hydrosoluble: <i>uric acid, ascorbic acid, glucose, cysteine, cysteamine, taurine, tryptophan, histidine, methionine, glutathione, plasma proteins</i>	
Liposoluble: <i>vitamin E, vitamin A, carotenoids, coenzyme Q, α-lipoic acid, bilirubin, thioredoxin, bioflavonoids, melatonin, lycopene</i>	
Chelating proteins: <i>transferrin, ferritin, caeruloplasmin, lactoferrin, haemopessin, albumin</i>	
ENZYMATIC	
Superoxide Dismutases (SOD): Cu/ZnSOD, MnSOD, CuSOD	
$2\text{O}_2^{\cdot -} + 2\text{H}^+ \longrightarrow \text{H}_2\text{O}_2 + \text{O}_2$	
Catalase (Cat)	
$2\text{H}_2\text{O}_2 \longrightarrow 2\text{H}_2\text{O} + \text{O}_2$	
Glutathione Peroxidases (GSH-Px)	
$2\text{GSH} + \text{H}_2\text{O}_2 \longrightarrow \text{GSSG} + \text{H}_2\text{O}$	
$2\text{GSH} + \text{ROOH} \longrightarrow \text{GSSG} + \text{H}_2\text{O} + \text{ROH}$	
Glutathione Reductases (GSH-Rd)	
$\text{GSSG} + \text{NADPH} + \text{H}^+ \longrightarrow 2\text{GSH} + \text{NADP}^+$	
$\text{NADP}^+ + \text{Glucose 6-Phosphate} \longrightarrow \text{NADPH} + \text{6-Phosphate Gluconic Acid}$	
Glutathione Transferases (GSH-Tr)	
It detoxifies electrophilic xenobiotics, unsaturated aldehydes (e.g. 4-hydroxynonenal, HNE) and hydroperoxides formed as secondary metabolites during oxidative stress	
Heme-oxygenase-I (HO-I)	
$\text{Heme} \longrightarrow \text{Bilirubin} + \text{CO} + \text{Fe}^{2+}$	

Mechanisms of Action and Chemical-Biological Interactions

Current Drug Therapy, 2009, Vol. 4, No. 3 161

one of the most protective protein. Similar huge differences have been calculated [17] for several other antioxidants. On this basis and using only the minimal, yet sufficient ozone dosage, it has become clear why we can safely use ozone as a medical drug and it will be further demonstrated how a precisely calculated ozone dose can be promptly tamed by the natural antioxidant system.

WAYS AND ROUTES OF OZONE ADMINISTRATION

After the preliminary clarification of the previous critical issue, the aim of this paper is to make a critical revision of the several ways currently used for ozone administration in patients, to evaluate the pros and cons of:

- 1) The major and minor ozonated autohemotherapy.
- 2) The extracorporeal circulation of blood against oxygen-ozone.
- 3) The intravenous infusion of ozonated physiological saline.
- 4) The intravenous infusion of ozonated water.
- 5) The quasi-total body exposure to oxygen-ozone.
- 6) The administration of the gaseous oxygen-ozone mixture *via* several routes: subcutaneous, intramuscular and intradiscal.
- 7) The intracavitary and intralesional administration of ozone.
- 8) The administration of oxygen-ozone *via* the colon-rectal route.

1) The Major and Minor Autohemotherapy (AHT)

These techniques represent the classical, old methods of blood ozonation. Major and minor AHT are referred only to a large (100-250 ml of blood) or to a small volume (5 ml) of blood, respectively. However, also their routes of administration are different: the former is intravenous and the latter is intramuscular. Moreover, there are fundamental differences regarding the preparation, the biochemistry, pharmacokinetic and the therapeutic aim. Both these procedures represent the paradigmatic example of ozone administration and they are also the best model for understanding the chemical reactions of ozone with blood components. The gas mixture is always composed of medical oxygen (about 95%) and ozone (about 5% or less). Oxygen is of medical grade and the gas mixture must be always sterilized by passing through an ozone-resistant filter (0.2 μm).

Is oxygen therapeutically important? Not in this case, even though oxygen must be used as the generator of ozone. As the 250 ml of the gas mixture contain no less than 95% oxygen, the pO_2 in the glass bottle containing 250 ml of anticoagulated (either with 3.8 % Na citrate or with heparin 10-20 IU/ml) blood, at a pressure of 700 mmHg allows the solubility of about 1.5 ml oxygen in the plasma, that is almost 7-fold higher than the physiological one, in the pulmonary veins. All haemoglobin is fully saturated with oxygen and exists as Hb_4O_8 only. However the relevance of the high oxygen tension is irrelevant because the infusion of the

ozonated blood into the donor takes about 20 min (about 12.5 ml/min) and within this period, it mixes with as many as 100 litres of venous blood with a pO_2 of about 40 mmHg. As a consequence the extra oxygen has a negligible value.

The real drug is ozone which appears to be 10-fold more soluble than oxygen in water, in relation to the experimental conditions [18]. The ozone dissolves very rapidly in the plasmatic water and immediately reacts with both hydro-soluble antioxidants (ascorbic acid: about 50 μM , uric acid: about 400 μM , reduced glutathione - GSH: about 6 μM) and polyunsaturated fatty acids (PUFA), mainly omega-6, bound to albumin. Normal human plasma contains about 5 mg/ml lipids but phospholipids and cholesterol in lipoproteins are not readily accessible to water-dissolved ozone [19]. Moreover, of the about 70 mg/ml proteins in plasma, about 40 mg are constituted by albumin (67 KDa) that contains one free cysteine (Cys34) and 17 pairs of disulfide bridges (S-S linkages) in its three homologous domains. During ozonation, the Cys-SH group of albumin could also allow the formation of Cys-SOH group, typical of sulfenic acid [20,21]. Thus, the potent antioxidant capacity of plasma is partly responsible for taming the strong oxidant properties of ozone. During its rapid decomposition, electrons donated to ozone by ascorbic acid transform it to dehydroascorbate, when the highest ozone concentration of the therapeutic range is used. Moreover, uric acid cooperates with ascorbic acid, but it is irreversibly oxidized to allantoin. The reaction with PUFA [22] occurs at the same time as shown in Fig. (1).

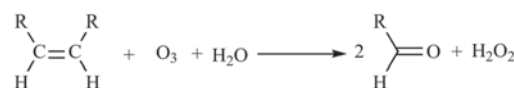


Fig. (1). Simplification of the PUFA ozonation process in aqueous environment.

This reaction has a fundamental importance because it generates two types of compounds, the first of which is H_2O_2 (included among Reactive Oxygen Species, ROS) with a very brief half-life and the second represented by a variety of aldehydes, relatively more stable.

Upon the variable characteristics of PUFA, several types of aldehydes are formed although HNE represents the main species. The free Cys(34) of albumin can be either readily oxidized or it can bind HNE. Moreover eleven accessible nucleophilic residues, constituted by Lys(199) and His(146) can also readily bind up to eleven HNE molecules. The small amount of cysteine, but especially free GSH in plasma can also act as an electron donor either being oxidized to sulfonates [23], or they can form an adduct with HNE at a slower rate constant than the nucleophilic albumin residues.

It is now clear that the ozone dose, calibrated against the antioxidant capacity of blood, is partly consumed by the readily available antioxidants and partly is used for generating the ozone messengers, H_2O_2 and HNE, necessary to elicit the biochemical reactions leading to therapeutic effects. It becomes understandable that a too small ozone dose will be totally quenched by antioxidants, while an excessive dose may damage blood cells.

For some time it has been known that the reducing compounds act as "sacrificial" molecules and albumin, besides being one of the most potent antioxidant [24], acts also as endogenous detoxifying agent of circulating reactive carbonyl species transporting them in the three hydrophobic pockets [25-27].

Physiological levels of transferrin, ceruloplasmin and metallothioneins prevent the formation of $\cdot\text{OH}$. Both uric acid and ascorbic acid are valuable scavenger of $\cdot\text{OH}$, $\text{O}_2\cdot^-$, O-NOO- and lipoperoxides [28-30]. H_2O_2 and peroxynitrite may also allow the formation of sulfenic acid in albumin [20,21] or induce its dimerization [31,32]. While allantoin is excreted, heavily oxidized albumin can be taken up by the reticulo-endothelial system without any negative effect owing to the large albumin pool and an intensive hepatic synthesis [33]. On the other hand, dehydroascorbate is reduced within a few minutes by recycling in the erythrocytes *via* the cooperation of thioredoxin, GSH-Rd and the continuous formation of reducing equivalents by glucose-6-phosphate dehydrogenase [9,10]. The scheme suggested by Parker (personal communication to V.B.) is very enlightening (Fig. 2).

During this rapid initial phase, ozone becomes extinct but it causes the formation of the messengers such as hydrogen peroxide and HNE as the most substantial aldehyde. The valid range of ozone dosages has been determined between 0.42 μM or 20 $\mu\text{g}/\text{ml}$ and 1.68 μM and 80 $\mu\text{g}/\text{ml}$ of gas per ml of blood) against the antioxidant capacity of blood (between 1.28 and 1.83 mmol/L plasma) [34]. At the usual therapeutical doses, ozone is unable to peroxidize phospholipids of erythrocytic membranes [5] and/or modify metahe-moglobin levels, to increase hemolysis or cause a leakage of K^+ and lactic dehydrogenase from erythrocytes, simply because the ozone dose is totally consumed in the plasma [35,36]. The electrophoretic mobility of erythrocytes as well as their osmotic fragility remain unmodified [36]. Moreover plasma levels of fibrinogen, cholesterol, triglycerides, HDL and LDL as well as enzymatic levels of superoxide dismutase, glucose-6-phosphate dehydrogenase, GSH-Px and GSH-Rd (U/g Hb) in erythrocytes do not vary after ozonation of blood within the therapeutic range [36]. Ozone dosages above 2.1 μM or 100 $\mu\text{g}/\text{ml}$ gas per ml of blood can slowly start to affect all of these parameters. On the other hand, very extensive alterations have been observed during ozonation of saline-washed human erythrocytes suspended in either saline or distilled water and it has been unfortunate that these artifactual and unphysiological studies have been performed reaching the wrong conclusion that ozone irreversibly damages erythrocytes [37,38]. However, these stud-

ies have been useful in proving the great importance of plasma antioxidants in neutralizing the deleterious ozone effects.

The next problem was to evaluate the biochemical and toxicological relevance of the most important ozone messengers: the peroxidative decomposition of the PUFA [39], on the basis of their varied and complex composition, lead to the release of a non-radical, reactive oxygen species such as hydrogen peroxide and a variety of lipid oxidation products (LOPs) such as lipoperoxides (LOO \cdot), alkoxy radicals (LO \cdot), lipohydroperoxides (LOOH), isoprostanes and a group of 4-hydroxylated-2,3-*trans*-alkenals of which the most quantitatively important is HNE. This aspect has been extensively reviewed by Barrera *et al.* [40] and Poli *et al.* [41].

As ozone dissolves into the water of plasma, hydrogen peroxide is generated within the first few minutes and, while it is partly quenched by antioxidants, it selects the PUFA as a preferred substrate. The establishment of a H_2O_2 gradient between the plasma and the cytoplasmic water of blood cells makes this oxidant a very early effector. Its concentration depends upon the ozone concentration but at a concentration of 1.68 $\mu\text{mol}/\text{ml}$ is no higher than 40 μmoles [42]. This concentration is very transitory because firstly, in the plasma, it is inactivated by antioxidants and secondly, by quickly passing through the cell membrane, undergoes dilution and further inactivation in the cytoplasmic water. Its intracellular concentration (at most 2-4 μmoles) has been assessed to be about 1/10 of the plasmatic one because it is quickly reduced to H_2O by free GSH, Cat and GSH-Px [4,6,42-46]. Its half-life is of about 1 sec and yet its intracellular concentration has a critical importance because activates the pentose cycle in erythrocytes [9,11], a tyrosine kinase in lymphocytes [9,47], and induces the release of growth factors from platelets [48]. Although the threshold is only of a few micromoles, it is physiologically important and means that an ozone dose below 0.21 $\mu\text{mol}/\text{ml}$ of gas per ml of blood, can be biologically ineffective because the ozone dose, hence the generated H_2O_2 is totally neutralized by the plasma antioxidants. In other words, the concept of a threshold helps to understand that a too low ozone dose may be ineffective (placebo effect), while a dose higher than the therapeutic one can be toxic. It is interesting that the ozonation process, characterized by the consumption of antioxidants, deeply differs whether it occurs in the plasma alone or in blood. Within the ozone therapeutic dose, when it occurs in the plasma, during the following 20 min the antioxidant capacity is reduced by 52 \pm 4 % and it does not recover, while in blood is reduced by 26 \pm 3% within one min, but it rapidly



Fig. (2). Pathways of the cellular reduction systems during oxidant insult.

Mechanisms of Action and Chemical-Biological Interactions

Current Drug Therapy, 2009, Vol. 4, No. 3 163

return to the initial value after 20 min. The rapid reconstitution of the antioxidant capacity was well shown to be due to the recycling process of dehydroascorbate to ascorbic acid, mainly operated by GSH and thioredoxin reductases [49,50]. Moreover the erythrocytes mass, *via* glucose-6-phosphate dehydrogenase activity, can continuously supply NADPH-reducing equivalents. Notably an increase of this enzyme has been determined in very young erythrocytes during ozone-therapy [9].

Given the toxicity of aldehydic LOPs, particularly regarding HNE [41], it is important to know their distribution, metabolism and fate.

HNE is a normally detectable molecule (0,7-1,0 μM) and on its own, is very unstable and toxic. At first it was surprising to observe that LOPs' levels (generated by ozonation of human plasma samples and measured as thiobarbituric acid reactive substances, TBARS) incubated *in vitro* at +37°C and pH 7.3, hardly declined during the next 9 hours indicating their stability in a acellular medium. On the other hand, the same samples, infused intravenously in the respective macular degeneration's patients, disappeared rapidly from the circulation with a half-life of 4.2 ± 1.7 min [51,52].

This result can be explained by the following five processes:

a) formation of albumin-HNE adducts

Assuming to ozonate 200 ml of blood with an ozone dose of 8 mg, the presence of about 5.4 g of albumin, particularly Cys(34) can form an adduct with HNE (see Fig. 3). Moreover HNE binds easily to the albumin nucleophilic groups [53].

b) dilution in the plasmatic and extravascular albumin pool

During the infusion of the ozonated blood into the patient, the albumin-HNE adducts will firstly dilute within the intravascular albumin pool represented by about 130 g albumin and then with the extravascular pool containing about 340 g albumin. Thus, in a total body pool of about 470 g albumin, the ozonated aliquot is less than 1%.

c) detoxification

This is operated very rapidly inside billions of cells by available GSH and at least three enzymes such as aldehyde dehydrogenase CYP450, aldose reductase and GSH-Tr [54,55].

d) excretion

HNE metabolized as mercapturic acid had been detected in urine and in bile after hepatic detoxification [56].

e) HNE as a signaling molecule

It is most interesting that traces of HNE or other alkenals-albumin adducts and sulfenic acid-albumin, in submicromolar concentrations, can act on a variety of organs as signaling molecules able to activate a number of biochemical pathways [53,57,58].

Processes a), b) and e) can explain that LOPs and particularly HNE, at submicromolar levels produced during calibrated blood ozonation will eventually inform cells from the bone marrow to the hypothalamus, of an acute oxidative stress. If this interpretation is correct, LOPs will have the role of late-acting messengers able to induce an upregulation of antioxidant enzymes and HO-I. Previous clinical data have shown that patients, if not already overwhelmed by the chronic oxidative stress, can express this positive response during prolonged ozonotherapy [9,59].

The major AHT has already proved to be very useful in vascular disorders complicated by ischemia because of an improved vasodilation in ischemic areas and an increased delivery of oxygenated blood. No evidence of side effects has ever been shown [7,9-11] but it remains impellent to perform a multicenter trial in thousands of patients for eliminating the persisting prejudice and convince sceptical angiologists that this approach is preferable to the intravenous infusion of prostanoids, which are expensive and often procure side effects.

By now, several million major AHT have been performed in many Countries, but one practical problem is the need to collect blood, to ozonize it *ex vivo* and to reinfuse it in the donor. Although the cost of the material is negligible and the whole procedure can be done in less than an hour, it needs to be performed at a hospital (or in a private clinic) and, when the patient is not auto-sufficient, a family's help is required. There is no legal problem for the physician to collect the patient's blood in a glass bottle provided that the blood is reinfused in the donor, who accept the procedure having signed an informed consent and with a compliance of almost 100%. The physician specialized in ozonotherapy cannot in any case infuse the blood in any other patient but this, to our knowledge, has never been done but it would infringe the law regulating blood transfusions. Collection of

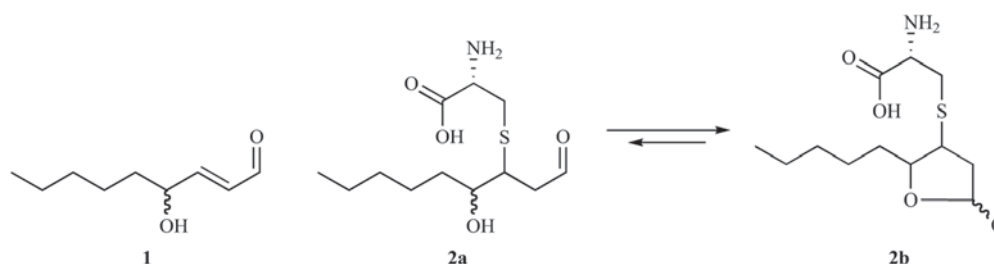


Fig. (3). HNE (1) and HNE-Cys-adducts (2a,2b). The lipid aldehyde HNE is an electrophile highly reactive with nucleophils (e.g. Cys, His and Lys).

pathology. Regretfully, improvement of our knowledge is slow because neither funds nor sponsors supporting this research have been so far available. Finally we have to denounce the use of common dialysis filters in the place of the appropriate GED in Asian countries. Although good clinical results have been claimed, dialysis filters are not ozone-resistant and can release toxic compounds in the patient's blood.

3) The Intravenous Infusion of Ozonated Physiological Saline

In 1994, it was demonstrated that ozonation of medical physiological saline (0.9% NaCl) with various ozone concentrations (50-70-100 $\mu\text{g/ml}$ ozone) induced at the same time formation of hydrogen peroxide and chemiluminescent effects indicating the generation of free radicals [4]. The production of H_2O_2 is progressive and by using an ozone concentration of 100 $\mu\text{g/ml}$ reached the value of 20 μM after 60 min ozone insufflation (Fig. 4).

Infusion of 250 ml of this solution in healthy volunteers caused considerable pain along the venous path of the infused arm after about 24 hours. This indicated that the solution has irritated the endothelium with the risk of a phlebitis and we were concerned that, besides H_2O_2 , a transitory formation of HOCl may be the noxious agent. Although chlo-

ride could be oxidized by ozone to perchlorate [73], the saline solution containing traces of Fe^{2+} may lead to the Fenton's reactions [74]:



Hypochlorous acid constitutes an inflammatory agent of the endothelium during an infusion, even at a concentration of 10 μM . Moreover, it may activate platelets and induce a microcoagulation. Although it is well known that ClO^- is physiologically produced by phagocytic cells and it is an efficacious bactericidal compound, it remains either confined in phagosomes or released in plasma near endothelial cells [75]. However, ClO^- is one of the most noxious reactive oxygen species (ROS) during a chronic inflammation.

It is unfortunate that the practice of using ozonated saline has become common in Russia and is widely used because it is inexpensive and less time-consuming than major AHT and simultaneously applicable to many patients. As it could be foreseen, physicians have started to use it also in Italy, Spain, Greece and Turkey. Ikonomidis *et al.* [76] have reported that they maintain the saline solution under a constant flow of ozone during transfusion but they warned that the maximum amount of ozone daily administered is usually 4-5 mg and

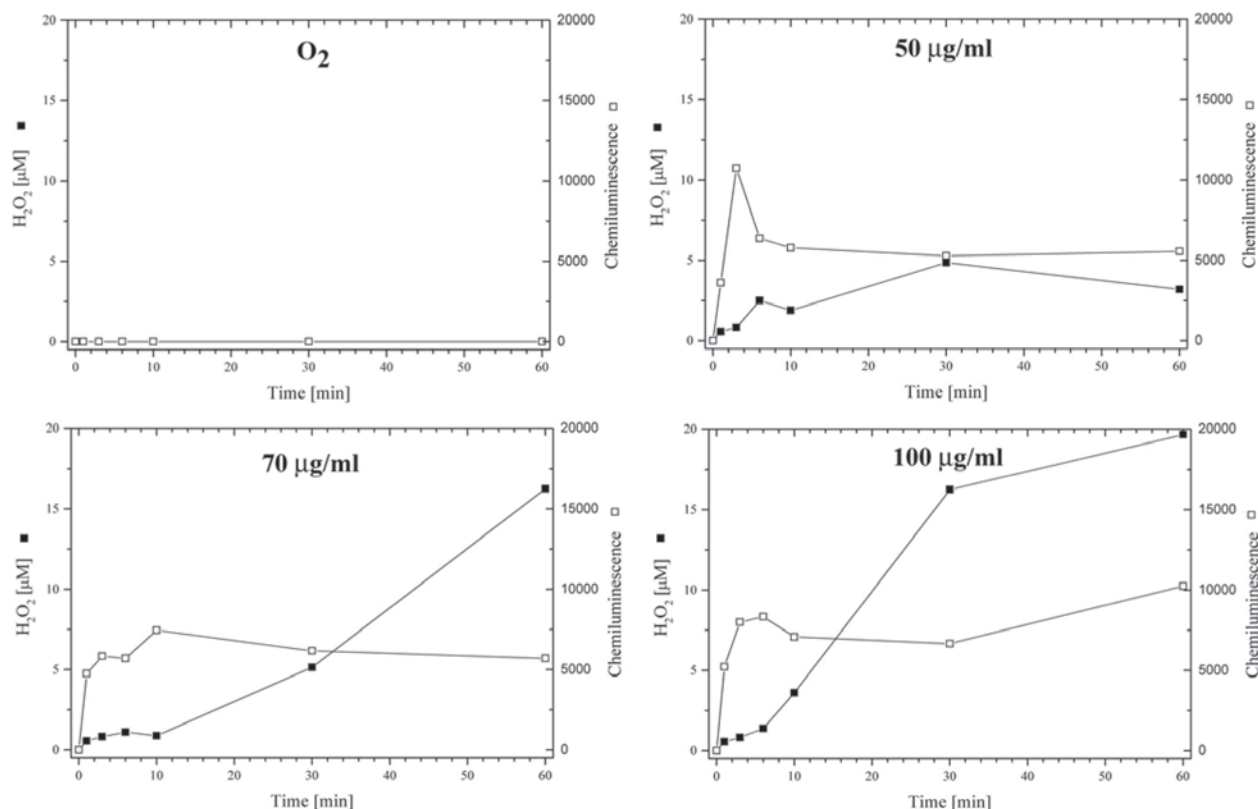


Fig. (4). Kinetics of the chemiluminescent signals and of the H_2O_2 production after exposing saline to oxygen alone or to three concentration of ozone (50, 70, 100 $\mu\text{g/ml}$ per ml of solvent).

Mechanisms of Action and Chemical-Biological Interactions

Current Drug Therapy, 2009, Vol. 4, No. 5 165

pathology. Regrettably, improvement of our knowledge is slow because neither funds nor sponsors supporting this research have been so far available. Finally we have to denounce the use of common dialysis filters in the place of the appropriate GED in Asian countries. Although good clinical results have been claimed, dialysis filters are not ozone-resistant and can release toxic compounds in the patient's blood.

3) The Intravenous Infusion of Ozonated Physiological Saline

In 1994, it was demonstrated that ozonation of medical physiological saline (0.9% NaCl) with various ozone concentrations (50-70-100 $\mu\text{g/ml}$ ozone) induced at the same time formation of hydrogen peroxide and chemiluminescent effects indicating the generation of free radicals [4]. The production of H_2O_2 is progressive and by using an ozone concentration of 100 $\mu\text{g/ml}$ reached the value of 20 μM after 60 min ozone insufflation (Fig. 4).

Infusion of 250 ml of this solution in healthy volunteers caused considerable pain along the venous path of the infused arm after about 24 hours. This indicated that the solution has irritated the endothelium with the risk of a phlebitis and we were concerned that, besides H_2O_2 , a transitory formation of HOCl may be the noxious agent. Although chlo-

ride could be oxidized by ozone to perchlorate [73], the saline solution containing traces of Fe^{2+} may lead to the Fenton's reactions [74]:



Hypochlorous acid constitutes an inflammatory agent of the endothelium during an infusion, even at a concentration of 10 μM . Moreover, it may activate platelets and induce a microcoagulation. Although it is well known that ClO^- is physiologically produced by phagocytic cells and it is an efficacious bactericidal compound, it remains either confined in phagosomes or released in plasma near endothelial cells [75]. However, ClO^- is one of the most noxious reactive oxygen species (ROS) during a chronic inflammation.

It is unfortunate that the practice of using ozonated saline has become common in Russia and is widely used because it is inexpensive and less time-consuming than major AHT and simultaneously applicable to many patients. As it could be foreseen, physicians have started to use it also in Italy, Spain, Greece and Turkey. Ikonomidis *et al.* [76] have reported that they maintain the saline solution under a constant flow of ozone during transfusion but they warned that the maximum amount of ozone daily administered is usually 4-5 mg and

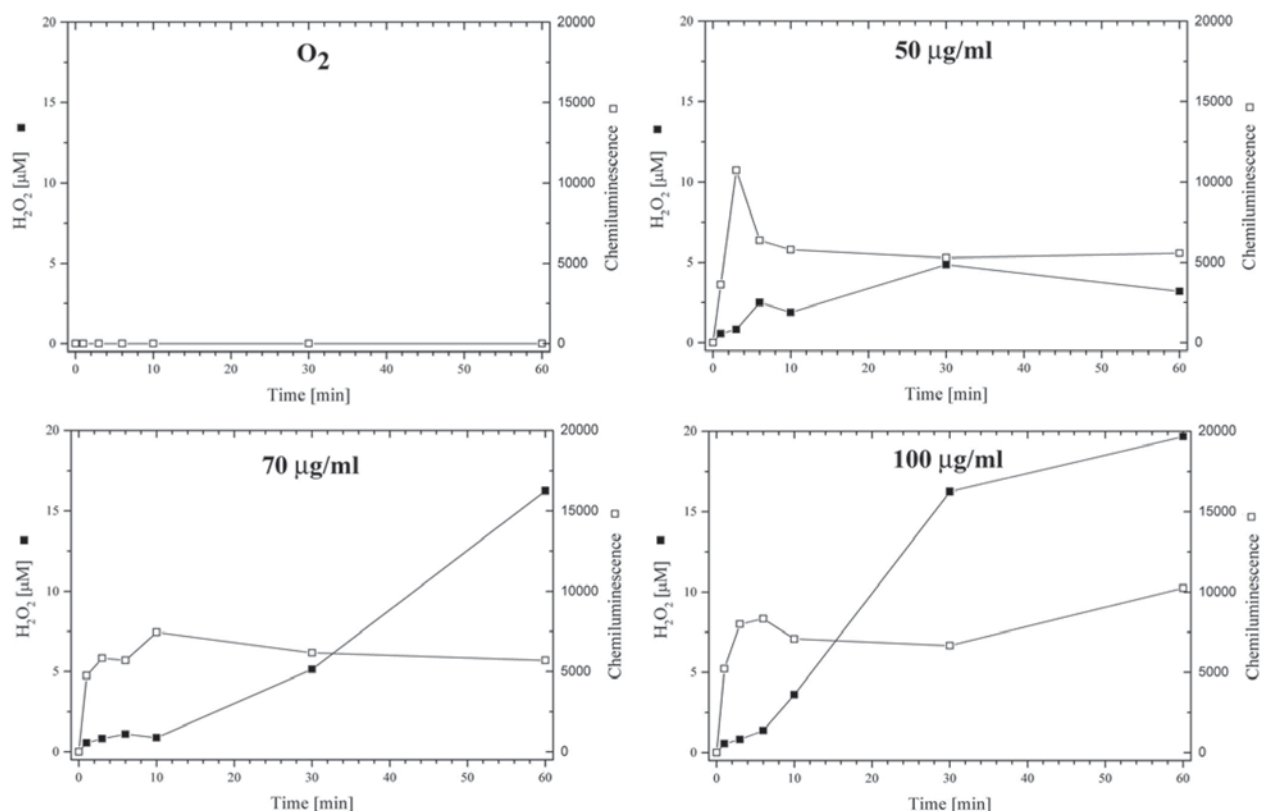


Fig. (4). Kinetics of the chemiluminescent signals and of the H_2O_2 production after exposing saline to oxygen alone or to three concentration of ozone (50, 70, 100 $\mu\text{g/ml}$ per ml of solvent).

should never exceed 8-10 mg. In their publication they also stated "if we exceed these rates, the over coagulation syndrome starts" and they strongly recommended to perform coagulation tests before starting therapy. These precautions reinforce our preliminary objection to this approach. Moreover, to the best of our knowledge, Russian physicians ozonize the saline with very low ozone concentrations (2-3 $\mu\text{g/ml}$) and this precaution may reduce toxicity. Clinical advantages have been claimed but results have never appeared in peer-reviewed journals; thus there is a serious concern that the advantage is due to a placebo effect not to be compared with the therapeutic advantage of properly ozonated blood. In comparison to the multiform effectors generated by ozone in blood, the ozonated saline appears as a palliative solution. Moreover, in contrast to ozonated saline, 0.03% (9 mM) H_2O_2 in isotonic glucose solution (5%), which does not contain traces of ClO^- have been safely infused [77]. Obviously, this solution should not be used in diabetic patients.

4) The Intravenous Infusion of Ozonated Water

Recently, a new technique based on a central vein infusion of ozonated water has been proposed and tested in a few cancer patients [78]. The definition of either "liquid polyatomic oxygen" infusion or "ozone in liquid form" is wrong because liquid ozone at ordinary pressure conditions exists at below -111.9°C , and it cannot be infused. Moreover, the term polyatomic oxygen is an euphemism because the corona-discharge method, one of the most effective procedures, practically generates only ozone.

Either ozone polymers (O_4) [79,80] or clusters (O_6 and superior) [81,82] can be generated. Thus, at this stage, it seems that the idea of superactive oxygen polymers/clusters must be further examined as well as if they have any therapeutic impact. Minimal details of the procedure have been reported [78] except the detail of ozonated water infusion *via* a Groshong type central venous catheter positioned in the patient's subclavian vein. It has been stated to perform a continuous administration for months of a mixture of "liquid polyatomic oxygen" useful for boosting the activity of some cytotoxic drugs. As we have already mentioned both ozone and oxygen are physically soluble in pure bidistilled water. When the gas mixture composed of oxygen (95%) and ozone (5%) is bubbled in pure water, ozone in relation to its relative pressure, temperature and solubility coefficient will dissolve as a gas in the water and will saturate it within 5-10 min up to 26% [83]. However ozone, even if kept in a tightly-closed ozone-resistant container spontaneously will decompose to oxygen during the following 11 hours at $+20^\circ\text{C}$. If the container is worn by a patient at the body temperature, ozone decays rapidly and the half-life is about 2.5 hours; this disadvantage, however, has not been mentioned. Provided that the gas mixture is dissolved in pure water at ordinary pressure, it is theoretically possible to infuse water-dissolved oxygen and ozone very slowly into the blood circulation. To the best of our knowledge, this technique had been firstly used by Belianin [84] in order to decrease the resistance of multiresistant mycobacteria in TBC patients. This is a technique for ozone administration probably less dangerous than the direct IV administration of gas correctly prohibited in 1984, owing to serious side effects and the risk

of oxygen embolism. However, it appears obvious that the infusion of "liquid polyatomic oxygen" will catastrophically freeze the blood and this term should be proscribed.

The proposed technique still present several disadvantages: firstly, there is no documented proof that a continuous deliver of solubilized ozone in pure water into the central venous system is more effective than the classical, practically risk-free, infusion of blood ozonated *ex vivo* into a cubital vein. Even in skilled hands, complications such as pneumothorax and sepsis are low but do happen. Venous thrombosis is also a risk always well emphasized by expert anesthesiologists. Noticeably, the patient must be informed about the implantation procedure, accept the procedure and sign an informed consent. The system can run smoothly only if the ozonetherapist procures the daily infusions, checks the pump programming, trains the patient and assiduously checks the safety of the system. Another crucial objection is that so far the usefulness of the solubilized ozone infusion without the integration of temozolomide (or other antitumoral agent) has not been demonstrated.

Although the direct intravenous injection of water-soluble oxygen and ozone is a very unusual and peculiar method of ozone administration, it deserves to be theoretically evaluated [9]. In order to prevent a pathological hemolysis (at the tip of the catheter, local hypotonicity cannot be lower than 100 mM NaCl) and in consideration that cancer patients undergo a chronic oxidative stress, we cannot infuse more than 360 ml (depending upon the body weight) of pure water during 24 hours. In other words, water can be cautiously infused at a rate of 0.50 ml/min. By using the currently available ozone generators, 360 ml of water at 20°C may dissolve no more than 9.1 mg ozone. However, as ozone decomposes rapidly at about 30°C , it is likely that, at best, we can deliver a negligible amount of oxygen and no more than about 6 $\mu\text{g/ml}$ ozone per minute. Thus the daily dose of ozone is below the average dose of 8.0 mg administered by ozonating 200 ml of blood *ex vivo* with an equal volume of gas containing 40 $\mu\text{g/ml}$ ozone. This reasoning questions the validity and usefulness of the direct infusion of water-soluble ozone in blood because ozone will immediately react with plasma components and it will never reach and kill neoplastic cells *in vivo*.

On the other hand, ozonated water can be rationally used if applied intratumorally, i.e., by using the Radial Expansible Retractor (RER) invented by one of us (D.M.) [85]. After placing the RER in the area of a glioblastoma multiforme (GBM), if the RER has been equipped with a silicon chamber supplied with an inlet and outlet tubings, it is possible to establish a constant flow of oxygen/ozone directed against the neoplasm. The outlet tubing leads to an ozone destructor to prevent air pollution. A second possibility is that ozonated water can be delivered very slowly directly into the neoplastic tissue for several days. Ozonation of medically pure, injectable water can be achieved by using the mixture oxygen-ozone under 2 atm pressure, possibly obtaining a concentration of 30-50 $\mu\text{g/ml}$ ozone physically dissolved in water. A third important possibility is the application of sterile ozonated olive or sesame oils of which we have good experience and have successfully used in deep necrotic ulcers [86].

Mechanisms of Action and Chemical-Biological Interactions

Current Drug Therapy, 2009, Vol. 4, No. 3 167

The main characteristic of these preparations is that ozone, while on itself very unstable, remains fixed and stable as a trioxolane between two carbons along the aliphatic chain (Fig. 5).

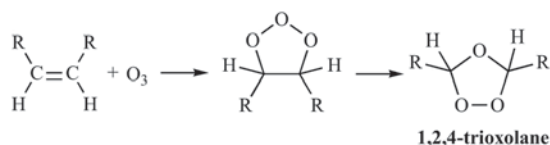


Fig. (5). Trioxolane formation by chemical reaction of ozone with PUFA in absence of water.

When ozonated oil, placed in a neoplastic tissue, comes in contact with the exudate, it slowly release peroxidic compounds that will easily kill GBM cells incapable to resist oxidation. As an example, application of ozonated oil could be performed during the night or when necessary.

GBMs are known to be the most aggressive and lethal tumors resistant to orthodox therapies. Whenever possible, after an initial surgery to remove the bulk of the tumor, the slowly but continued administration of ozonated water or of ozonated oil will release of ozone in swollen cells and since 1980 [87] and our experience of cancer cells kept *in vitro* under ozone, leaves little doubt of a progressive killing of glioblastoma cells until we reach normal vascularized tissue where ozone activity is rapidly neutralized. The use of ozonated water and oil appears greatly advantageous over the use of ozone as a gas because not only the atonic water has a swelling effect but it allows the release of ozone inside the cells and prevents also the risk of polluting the surrounding air. Moreover the successive use of temozolomide and a vaccine against epidermal growth factor receptor variant III (EGFRvIII) [88,89] may lead to a prolonged remission, if not a cure.

The same procedure can be evaluated in patients with cerebral abscesses, notoriously difficult to be treated owing to antibiotic resistance and to the local difficulty of achieving an effective bactericidal activity.

A very much experimented and useful application is the topical use of pure ozonated water in chronic ulcers (diabetic foot), decubitus and many other cutaneous and mucosal infections. This is a problem of great socio-economical interest because millions of patients suffer of chronic cutaneous infections that never heal and cause pain and depression. An extensive review on the topical use of gaseous ozone and derivatives as anti-infective agents is being published [86]. Finally, a great progress has been achieved by the topical application of gaseous ozone, ozonated water and oil derivatives in dentistry [90,91].

5) The Quasi-Total Body Exposure to Oxygen-Ozone

An extensive experience in treating localized chronic infections or ulcers in the skin has been gained during the last 25 years by exposing the lesion to oxygen-ozone in a totally humidified area enclosed in a tightly-closed polyethylene bag. The so-called "bagging system" depending upon the availability of an ozone generator with an aspirating pump can be dynamic in the sense that there is a continuous

input and a corresponding gas output where ozone is decomposed by a suitable destructor. The environmental air must never be contaminated with ozone. More frequently, the system is static with the bag filled with gas for about 20 min, a period sufficient to disinfect the lesion and enhance healing. Also in this occurrence, ozone must be aspirated and destroyed. This procedure is valuable for a variety of infected and necrotic lesions caused by either ischemia, or venous stasis or diabetes, or trauma, burns and bed sores. In this cases, ozone concentration can be as high as 70 µg/ml because that is needed for highly infected lesions and because ozone at about 30°C quickly decomposes. As soon as the lesions progressively improve lower ozone concentrations are used to enhance cell proliferation and healing. As no damage has been observed on the surrounding normal skin, it has been obvious to examine the value of ozone in other pathologies with generalized lesions such as atopic dermatitis, eczema, psoriasis and seriously advanced lipodystrophy.

As a large polyethylene bag is only a temporary solution, suitable ozone-resistant cabins with an ergonomic seat and back rest have been built where the body of the patient except than the head remains perfectly insulated inside, without risk of breathing ozone. Moreover, a thermostatic controlled system allows to regulate the internal temperature (from 36 to 42°C) and saturated water vapor, essential for maximal ozone activity. The patient can sit inside the chamber for 20-30 min but before opening the cabin, the internal gas (maximal ozone concentration is no more than 1 mg/L) is again aspirated and destroyed. This is indeed an interesting system not only for treating ample cutaneous lesions, but also for treating systemic diseases. As usual ozone is not absorbed through the skin because it immediately reacts with the water film covering about 1.7 m² of the skin surface. Sweat and sebum secreted compounds enhance the ozone reactions and it was of great interest to examine the modification of several metabolic parameters [92,93]. After 20 min permanence in the cabin, body weight decreased of almost 1 kg and systolic pressure also decreased of about 10 mmHg. Pressure of either oxygen or PvCO₂ markedly increased or decreased, respectively, indicating oxygen absorption through the skin. While ozone was not absorbed, peroxidation compounds were and doubled in the venous blood. On the basis of evaluation of several other parameters, the conclusion was reached that the quasi-total body exposure to oxygen-ozone mixture involves a reaction of the whole body and it represents a very promising procedure for modifying the biological response in different pathologies. We have experimented it ourselves and many physicians have volunteered to test the system and conclusions have been enthusiastic. The treatment is simple, inexpensive to perform and not-invasive (no venous punctures are required) and does not involve the handling of potentially infected blood, an aspect much appreciated by medical personnel. It requires only to be well-organized with a small room where to undress and a room for a comfortable one-hour rest for the patient with a final shower, if desired. No side effects have been noted and everyone who has tested the system reports a feeling of well-being next day. This approach for which an extensive report was published in 2005 [94], may therefore will compete with major AHT, EBOO and certainly other administration ways of gaseous ozone, like the rectal insufflation.

6) The Administration of the Gaseous Oxygen-Ozone Mixture Via Several Routes

a) The gaseous mixture oxygen-ozone collected with an ozone-resistant syringe can be injected into various body compartments. As an example, the subcutaneous (SC) injection of several small gas volume (1-2 ml) with an ozone concentration between 2-3 µg/ml of gas has been amply used for the treatment of lipodistropia (cellulitis). On the whole, no more than 40 small injections (total volume ~ 80 ml) should be injected for each treatment. At the end of the session, a gentle massage for about 10 min should enhance the lipolytic activity and accelerate the gas reabsorption. The treatment is effective, atoxic and the risk of a pulmonary embolism due to oxygen is minimal provided to avoid injection of over 80 ml gas in about 40 SC areas [10,11]. Indeed larger volumes (up to 250 ml) are dangerous and have caused three deaths in Italy.

b) The intramuscular (IM) injection of ozone as a substitute of major AHT is not performed today because a minimal 4 mg ozone should be injected by using 200 ml gas with an ozone concentration of 20 µg/ml that would be dangerous and painful.

On the other hand, the IM injections in the paravertebral muscle of about 10-20 ml of the gas mixture (with an ozone concentration ranging from the initial 15 up to 25 µg/ml, total ozone dose of 300-500 µg) are in wide use. The route of infiltration of the gas is located in the paravertebral muscle, about two cm bilaterally to the spinous process, just above the transiliac line, points useful for identifying the L4 spinous process in case of a hernial disk between L4-L5. The procedure has been defined as a "chemical acupuncture" or the indirect approach [95]. This methodology is hardly fifteen years old but, by now, many patients have been treated in Italy, China, India, Spain and Germany during an episode of low back-ache.

It is an easy approach consisting in one or two injections of 5-10 ml of gas per site. The ozone concentration normally must not exceed 20 µg/ml because it is painful except when, after several injections, the pain threshold has markedly gone up. At first, it is wise to test the patient's reactivity with an injection of sterile saline and then start with 10 µg/ml of ozone. The injection must be done very slowly into the trigger points corresponding to the metamers of the herniated disk. The length of the needle varies from gauge 22 to 25, depending on the patient's obesity. Usually, two symmetrical injections (total dose 10-20 ml gas with at most 200-400 µg ozone) repeated twice per week for about 5-6 weeks (10-12 sessions) are sufficient. If not, the patient is unresponsive to this approach. This point remains controversial because some ozonetherapists continuous treatments up to 30 sessions. The pain at first elicited with an ozone concentration of 20 µg/ml tends to subside because of a progressive elevation of the pain threshold. In such a case, the ozone concentration can be increased up to 25 µg/ml. It appears that the stimulation of nociceptors, hence of a tolerable and transitory pain is an essential requirement for achieving the final therapeutic effect. Indeed, the patient must be reminded that "no pain, no gain".

Indeed the injection of oxyge/ozone elicits a fairly sharp pain lasting a few minutes and the injection must be done very slowly to avoid any risk of embolism. Serious adverse effects, such as sudden hypotension, bradycardia, mydriasis, intense perspiration and cardiac arrest (vasovagal reflex) can be also avoided by a very slow injection.

An important question is: how does intramuscularly injected ozone work? The gas infiltrates the muscle and after 24 hours some gas bubbles (residual oxygen) move towards the vertebral canal, as can be radiologically seen. It was postulated that ozone will reach the site of the herniated material and will lyse it. This is an untenable idea: ozone is water-soluble and it dissolves so quickly into the interstitial water of the muscle that within 20-40 sec will generate a gradient of ROS and LOPs able to inhibit amyelinic fibres (C-nociceptors), which are able to elicit the elevation of pain threshold and an antalgic response *via* the descending antinociceptive system. The introduction of the needle, reinforced by the pressure of the gas, induces a prolonged stunning of nociceptors due to ROS and LOPs. It is known that an algic skin and muscle stimulation can reduce pain through the mechanism of diffuse noxious inhibitory control (DNIC) [96]. That is why the needle, in combination with ROS, LOPs and oxygen pressure can be translated into a chemical acupuncture. This mechanism is likely correct because too low ozone concentration (3-10 µg/ml) or small gas volumes (1-2 ml) are ineffective, whereas too high concentrations or excessive gas volumes can cause lipothymia. It is unclear whether pre-infiltration with an anaesthetic reduces the effect of ozone but probably it is counterproductive.

In conclusion, the probable mechanisms [95] playing a role are the following:

- i) release of endorphins and endocannabinoids blocks transmission of the noxious signal to the thalamus and cortex;
- ii) hypostimulation (elevation of the activation threshold) linked to the oxidative degeneration of C-nociceptors. ROS and LOPs may act as capsaicin;
- iii) activation of the descending antinociceptive system;
- iv) simultaneous psychogenic stimulation of the central analgesic system induced by the gas injection (elicitation of a placebo effect);
- v) the localized oxygenation and analgesia are most important because they permit muscle relaxation and vasodilation, thus a reactivation of muscle metabolism, by favoring oxidation of lactate, neutralization of acidosis, increased synthesis of ATP, Ca²⁺ reuptake and absorption of edema.

The reader may be interested to know that after performing about a dozen IM injections, up to 75% of patients have a good and long-lasting response. It appears that this approach is far more valid than either the peridural injections of dexamethasone or the paravertebral injection of 0.25% bupivacaine.

c) The other relevant breakthrough regarding the frequent problem of back-ache is the direct injection of the gas mix-

Mechanisms of Action and Chemical-Biological Interactions

Current Drug Therapy, 2009, Vol. 4, No. 3 169

ture (~ 2-6 ml of gas with an ozone concentration up to 25-30 µg/ml; ozone total dose 50-180 µg) into the nucleus pulposus related to the hernial disk site [11]. During lifetime, low-back pain is a very disturbing symptom that can affect, at least for a while, up to about 80% of the world population [97,98]. Luckily, in most cases physical therapies (exercises, manipulation therapy) can solve the problem [99], but if a hernial disk is present it must be removed with the least invasive procedure. One of the most obvious mechanism of action is that ozone dissolves in the interstitial water of the nucleus and immediately reacts, generating a cascade of ROS among which H₂O₂ and [•]OH, that is a most reactive radical. Indeed, this radical is likely to react with carbohydrates and amino acids composing proteoglycan, a major component of the nucleus pulposus, leading to its breakdown [100-102]. Consequently, reabsorption of hydrolytic products and water appears to lead to progressive shrinkage and disappearance of the herniated material that is responsible for radicular pain. Detection of free radicals is difficult because they are very reactive and short lived. Indeed, the half-life at 37°C of the [•]OH radical is extremely short, equivalent to about 10⁻⁹ sec. Electron Spin Resonance (ESR) is the only analytical technique currently capable of directly measuring free radicals. The combined technique of ESR and spin trapping is highly selective and sensitive for the detection of free radicals. A specific study [103] has shown that during the exposure of a nucleus pulposus gel material to oxygen-ozone mixture, the hydroxyl radical is the unique radical species produced. This radical is so reactive that can start a chain reaction leading to oxidation and degradation of proteoglycans able to explain the rapid disappearance of herniated material.

There is no doubt that hydroxyl radicals can cause the depolymerization of hyaluronic acid, synovial fluid as well as collagen and that the degradation could be inhibited by free radical scavengers. Moreover, this is likely to happen because the natural antioxidant system, composed of hydrophilic, lipophilic substance and enzymes [104], is hardly present in the discal material. However, also in this case there is evidence of the ozone paradox [11]: although hydroxyl radicals can degrade the degenerated material and reduce pressure, they surprisingly exert a rapid "anti-inflammatory action", particularly because only a few ml of gas can be introduced inside the nucleus pulposus and some of the gas invades the intraforaminal space. This may mean that ozone rapidly blocks inflammatory reactants and stimulates the "restitutio ad integrum". What is even more surprising is that this change remains stable (unlike corticosteroids) and it does not necessarily coincide with the disappearance of the herniated material. In fact, CAT or NMR controls 5 months after treatment in 612 patients showed that the hernia disappeared in 226 (37%), was reduced in 251 (41%) and was unmodified in 135 (22%). After another 5 months, CAT/NMR controls were again performed in 200 (of 251) patients in whom the hernia was reduced: a further reduction and improvement was noted in 44 patients (22%). In 120 patients (of 135) in whom the hernia was unmodified, there was an improvement in 11.6% [105]. Thus, the ozone effect is deployed in successive phases: there is an initial rapid change, probably with disappearance of edema and improvement of circulatory and metabolic conditions, followed

by a stasis and then a further improvement possibly due to release of TGFβ1 and bFGF [106] favoring the reorganization of the residual nucleus pulposus with incipient fibrosis. Moreover, ozone may inactivate proinflammatory cytokines, inhibit proteinases and cyclooxygenase-2 thus blocking the synthesis of prostaglandin E₂. All of these surprising aspects have contributed to formulate the concept of the ozone paradox [107].

A few problems have been reported. In young patients, it is often very difficult to introduce more than 1-2 ml of gas inside the nucleus pulposus, so that the gas is mostly released into the intraforaminal space. In these cases, a preliminary aspiration of the nucleus followed by the gas introduction might improve the result. Apparently, the intraforaminal administration of gas yields good results even in the case of sclerotic hernias [108]. Side effects are very rare: one patient had a transient lipothymia and one reported by Alexander *et al.* [109] presented amaurosis fugax (bilateral blindness which reversed after about 24 h) after cervical discolysis in a young athlete.

Dr. Kieran J. Murphy, an interventional neuroradiologist at the University of Toronto, at the annual meeting of the Society of Interventional Radiology in San Diego, CA (March 2009), reported a study performed in more than 8000 patients with herniated disk injected with ozone intradiscally injected (unpublished data). Results have been so good (about 80% success rate) to predict that the procedure so simple, atraumatic, safe, and atoxic will become standard in the US within the next five years. Surprisingly some patients have no longer pain 30 min after the injection suggesting the rapid effect of ozone in releasing the pressure and inflammation on the nerve root. He acknowledged that the procedure had been discovered in Italy, where more than 20,000 patients have been already treated [110], not to count many thousands in China, India and Spain. Patients treated with ozone fare much better than those treated with steroids [111,112].

7) The Intracavitary and Intralesional Administration of Ozone

Although the intraperitoneal (IP) administration has been advocated in several pathologies, such as chronic hepatitis B and C in nephropathic patients under peritoneal dialysis [10,11], and in ovarian carcinosis with peritoneal involvement, so far it has not been possible to perform a study in patients. During peritoneal dialysis, when a patient has already a medical grade silastic catheter, would be simple to try the concept. However, clinicians are skeptical and are afraid of a potential ozone toxicity. Schulz *et al.* [113] have recently shown that 6 rabbits over 14 implanted with VX2 carcinoma HNSCC tumor cells can be "cured" after the IP injection for 5 consecutive days of 12 mg of ozone. The results are interesting, but also in the past the "cure" had been observed in experimental mice with IL-2, tissue-infiltrating lymphocytes (TIL) and angiogenesis inhibitors, drugs that in clinical trials have failed to solve the cancer problem. Indeed, in a letter the question posed was: "Does ozone really cure cancer?" [114]. In fact, after performing prolonged cycles of major AHT in preterminal metastatic cancer patients, results have been disappointing [115]. How-

ever, peritoneal carcinosis and mesothelioma, besides being almost refractory to chemo-radiotherapy remains to be evaluated, by using ozonated water and oil as well as gas well retained in the peritoneal and pleural cavities. In preliminary experiments in rabbits, an intraperitoneal injection of 250 ml of gas, with an ozone concentration of 20 µg/ml has not procured any noxious effect. Also a localized melanoma could be destroyed by direct administration of ozone but unfortunately ozone, intended as a direct cytotoxic agent, can never reach metastatic cancer cells. The cytotoxic effect of ozone in GBM remains to be ascertained.

8) The Administration of Oxygen-Ozone Via the Colon-Rectal Route

This problem has been extensively discussed [116] because it is an easy and rapid way of administering ozone. It has the additional advantage that the patient could self-administer ozone at home under the physician's advice. There are however several caveats to bear in mind. Firstly, while oxygen is partly absorbed, it has a negligible value. Ozone cannot be absorbed by the colon-rectal mucosa because it immediately reacts with the heterogeneous luminal content (e.g. feces, mucoproteins, immunoglobulins). A variety of peroxidation compounds detected in portal blood were absorbed as it was shown in rabbits after preventing the loss of the administered gas [117]. This route is widely used at Cuba and in Russia: clinical data have been published [118-121], but results need to be confirmed by other institutions. The insufflated dose of 6-12 mg ozone (200-400 ml of gas with an ozone concentration below 30 µg/ml) is known but it always uncertain the quantity of ozone effectively acting in the gut. Ozone concentration higher than 30 µg/ml can be mutagenic on the mucosal cells especially if the patient has made a clyisma before the treatment. Thus the rectal route remains unreliable and it was found less effective than major AHT [119].

CONCLUSION AND PERSPECTIVES

The evaluation of the mechanism of action of ozone in biological fluids has allowed to precisely clarify how ozone works and why it is not toxic when used within the therapeutic range. The versatility of ozone applications is impressive and today ozonotherapy can be performed using several different modalities. Besides the old but still quite valid methods of major and minor autohemotherapy, other options such as the quasi-total body exposure to oxygen-ozone and the EBOO have been developed and evaluated. In non-diabetic patients with precarious venous access, as a blood substitute, the glucose-peroxide solution, which represents a form of biooxidative therapy with a clear rationale and the advantage of being inexpensive and potentially useful to millions of people without medical assistance can be profitably used. Although all of these procedures must be controlled and supervised by physicians expert in ozonotherapy, a few of them are amenable to be used at home by the patient. Ozone must never be breathed but, if the dose is adapted to the potent antioxidant capacity of body fluids, the above described methods offer flexible and useful therapeutic advantages. Whenever necessary, it is possible to combine these different approaches. The central aim of ozonotherapy is to give a precise, atoxic shock to an organism which for various rea-

sons has gone astray. In agreement with the "hormesis" concept, repeated, small shocks will readjust several biological functions by means of many messengers (ROS, LOPs and autacoids generated by ozone) delivered by circulating blood to the whole body. The term "therapeutic shock" has been coined to symbolize the possibility of reactivating the natural positive capabilities to restore health or, in better words, to stimulate the "vis medicatrix naturae".

The simultaneous induction of an acute and precisely calculated oxidative stress on different areas such as blood, the skin and gut mucosal system can result in a more comprehensive and perhaps synergistic response of the body defense system. Indeed, chronic diseases must be attacked from different angles and we have evidence that the stimulation of several biochemical pathways in different organs can be therapeutically beneficial.

Some Nations in the world still do not allow the practice of ozonotherapy either for negligence, prejudice or because it is dangerously performed by incompetent quacks without medical qualifications. As a consequence it is necessary to establish precise regulations, the first of which is that only physicians, after an appropriate university qualification, can perform it. A further significant step forward will be done by performing randomized clinical trials definitively proving the advantage of ozonotherapy over orthodox medications in vascular disorders. Much remains to explore in other pathologies where ozonotherapy today can only used as a supporting therapy. An obvious remark is that, against the minimal cost of ozone, the expenses of medical assistance and drug development increases every day and normally a decade is necessary before a drug becomes of practical use. The review aims to clarify to inexperienced clinicians not only the specific medical advantages of this approach but also the possibility of extending its use in poor countries. It remains deplorable that Health Authorities do not sponsor further studies and an application of this approach in every hospital.

ACKNOWLEDGEMENT

The Authors gratefully acknowledge the gratuitous revision of the English by Mrs. Helen Carter.

REFERENCES

- [1] Stoker G. The surgical uses of ozone. *Lancet* 1916; 188: 712.
- [2] Wolff HH. Die Behandlung peripherer Durchblutungsstörungen mit Ozon. *Erfahr Hk* 1974; 23: 181-4.
- [3] Jonas WB. Alternative medicine - learning from the past, examining the present, advancing to the future. *JAMA* 1998; 280: 1616-8.
- [4] Bocci V, Valacchi G, Corradeschi F, et al. Studies on the biological effects of ozone: 7. Generation of reactive oxygen species (ROS) after exposure of human blood to ozone. *J Biol Regul Homeost Agents* 1998; 12: 67-75.
- [5] Shinriki N, Suzuki T, Takama K, et al. Susceptibilities of plasma antioxidants and erythrocyte constituents to low levels of ozone. *Haematologia* 1998; 29: 229-39.
- [6] Bocci V, Aldinucci C. Biochemical modifications induced in human blood by oxygenation-ozonation. *J Biochem Mol Toxicol* 2006; 20: 133-8.
- [7] Bocci V. Scientific and medical aspects of ozone therapy. *State of the art. Arch Med Res* 2006; 37: 425-35.
- [8] Bocci V. The case for oxygen-ozonotherapy. *Br J Biomed Sci* 2007; 64: 44-9.
- [9] Bocci V, Borrelli E, Travagli V, Zanardi I. The ozone paradox: Ozone is a strong oxidant as well as a medical drug. *Med Res Rev* 2009; 29: 646-82.

Mechanisms of Action and Chemical-Biological Interactions

Current Drug Therapy, 2009, Vol. 4, No. 3 171

- [10] Bocci V. Oxygen-Ozone therapy. A critical evaluation. Dordrecht, The Netherlands, Kluwer Academic Publishers 2002.
- [11] Bocci V. Ozone. A new medical drug. Dordrecht, The Netherlands, Springer 2005.
- [12] Calabrese EJ. Paradigm lost, paradigm found: the re-emergence of hormesis as a fundamental dose response model in the toxicological sciences. *Environ Pollut* 2005; 138: 379-411.
- [13] Nakao A, Sugimoto R, Billiar TR, McCurry KR. Therapeutic antioxidant medical gas. *J Clin Biochem Nutr* 2009; 44: 1-13.
- [14] Bell ML, McDermott A, Zeger SL, Samet JM, Dominici F. Ozone and short-term mortality in 95 US urban communities, 1987-2000. *JAMA* 2004; 292: 2372-8.
- [15] Jerrett M, Burnett RT, Pope CA 3rd, *et al.* Long-term ozone exposure and mortality. *N Engl J Med* 2009; 360: 1085-95.
- [16] Last JA, Gohil K, Mathrani VC, Kenyon NJ. Systemic responses to inhaled ozone in mice: cachexia and down-regulation of liver xenobiotic metabolizing genes. *Toxicol Appl Pharmacol* 2005; 208: 117-26.
- [17] Bocci V. Is it true that ozone is always toxic? The end of a dogma. *Toxicol Appl Pharmacol* 2006; 216: 493-504.
- [18] Battino R, Rettici TR, Tominaga T. The solubility of oxygen and ozone in liquids. *J Phys Chem Ref Data* 1983; 12: 163-78.
- [19] Cross CE, Reznick AZ, Packer L, Davis PA, Suzuki YJ, Halliwell B. Oxidative damage to human plasma proteins by ozone. *Free Radic Res Commun* 1992; 15: 347-52.
- [20] Carballeda S, Radi R, Kirk MC, Barnes S, Freeman BA, Alvarez B. Sulfenic acid formation in human serum albumin by hydrogen peroxide and peroxynitrite. *Biochemistry* 2003; 42: 9906-14.
- [21] Carballeda S, Alvarez B, Turell L, Botti H, Freeman BA, Radi R. Sulfenic acid in human serum albumin. *Amino Acids* 2007; 32: 543-51.
- [22] Pryor WA, Das B, Church DF. The ozonation of unsaturated fatty acids: aldehydes and hydrogen peroxide as products and possible mediators of ozone toxicity. *Chem Res Toxicol* 1991; 4: 341-8.
- [23] Enami S, Hoffmann MR, Colussi AJ. Ozone oxidizes glutathione to a sulfonic acid. *Chem Res Toxicol* 2009; 22: 35-40.
- [24] Larini A, Bocci V. Albumin is the most effective antioxidant during human plasma and blood ozonation. *Rivista Italiana di Ossigeno-Ozonoterapia* 2004; 3: 15-24.
- [25] Sugio S, Kashima A, Mochizuki S, Noda M, Kobayashi K. Crystal structure of human serum albumin at 2.5 Å resolution. *Protein Eng* 1999; 12: 439-46.
- [26] Aldini G, Gamberoni L, Orioli M, *et al.* Mass spectrometric characterization of covalent modification of human serum albumin by 4-hydroxy-trans-2-nonenal. *J Mass Spectrom* 2006; 41: 1149-61.
- [27] Aldini G, Vistoli G, Regazzoni L, *et al.* Albumin is the main nucleophilic target of human plasma: A protective role against proatherogenic electrophilic reactive carbonyl species? *Chem Res Toxicol* 2008; 21: 824-35.
- [28] Van der Vliet A, O'Neil CA, Eiserich JP, Cross CE. Oxidative damage to extracellular fluids by ozone and possible protective effects of thiols. *Arch Biochem Biophys* 1995; 321: 43-50.
- [29] Halliwell B. Antioxidants in human health and disease. *Annu Rev Nutr* 1996; 16: 33-50.
- [30] Kermani S, Ben-Jebria A, Ultman JS. Kinetics of ozone reaction with uric acid, ascorbic acid, and glutathione at physiologically relevant conditions. *Arch Biochem Biophys* 2006; 451: 8-16.
- [31] Bocci V. Biological behavior of rabbit ¹²⁵I-albumin polymers. *Arch Biochem Biophys* 1967; 120: 621-7.
- [32] Ogasawara Y, Namai T, Togawa T, Ishii K. Formation of albumin dimers induced by exposure to peroxides in human plasma: a possible biomarker for oxidative stress. *Biochem Biophys Res Commun* 2006; 340: 353-8.
- [33] Bocci V. Catabolism of plasma proteins. In: Allison AC Ed, Structure and function of plasma proteins. New York, Plenum Press 1976; 163-88.
- [34] Miller NJ, Rice-Evans C, Davies MJ, Gopinathan V, Milner A. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clin Sci* 1993; 84: 407-12.
- [35] Travagli V, Zanardi I, Bocci V. A realistic evaluation of the action of ozone on whole human blood. *Int J Biol Macromol* 2006; 39: 317-20.
- [36] Travagli V, Zanardi I, Silvietti A, Bocci V. A physicochemical investigation on the effects of ozone on blood. *Int J Biol Macromol* 2007; 41: 504-11.
- [37] Goldstein BD, Balchum OJ. Effect of ozone on lipid peroxidation in the red blood cell. *Exp Biol Med* 1967; 126: 356-8.
- [38] Cataldo F, Gentilini L. Chemical kinetics measurements on the reaction between blood and ozone. *Int J Biol Macromol* 2005; 36: 61-5.
- [39] Esterbauer H, Zollner H, Lang J. Metabolism of the lipid peroxidation product 4-hydroxynonenal by isolated hepatocytes and by liver cytosolic fractions. *Biochem J* 1985; 228: 363-73.
- [40] Barrera G, Toaldo C, Pizzimenti S, *et al.* The role of PPAR ligands in controlling growth-related gene expression and their interaction with lipoperoxidation products. *PPAR Res* 2008; 2008: 524671.
- [41] Poli G, Schaur RJ, Siems WG, Leonarduzzi G. 4-Hydroxynonenal: a membrane lipid oxidation product of medicinal interest. *Med Res Rev* 2008; 28: 569-631.
- [42] Valacchi G, Bocci V. Studies on the biological effects of ozone: 11. Release of factors from human endothelial cells. *Mediators Inflamm* 2000; 9: 271-6.
- [43] Bocci V, Valacchi G, Corradeschi F, Fanetti G. Studies on the biological effects of ozone: 8. Effects on the total antioxidant status and on interleukin-8 production. *Mediators Inflamm* 1998; 7: 313-7.
- [44] Antunes F, Cadenas E. Estimation of H₂O₂ gradients across biomembranes. *FEBS Lett* 2000; 475: 121-6.
- [45] Stone JR, Collins T. The role of hydrogen peroxide in endothelial proliferative responses. *Endothelium* 2002; 9: 231-8.
- [46] Stone JR, Yang S. Hydrogen peroxide: a signaling messenger. *Antioxid Redox Signal* 2006; 8: 243-70.
- [47] Bocci V, Luzzi E, Corradeschi F, Paulesu L, Di Stefano A. Studies on the biological effects of ozone: 3. An attempt to define conditions for optimal induction of cytokines. *Lymphokine Cytokine Res* 1993; 12: 121-6.
- [48] Bocci V, Valacchi G, Rossi R, *et al.* Studies on the biological effects of ozone: 9. Effects of ozone on human platelets. *Platelets* 1999; 10: 110-6.
- [49] Mendiratta S, Qu ZC, May JM. Erythrocyte ascorbate recycling: antioxidant effects in blood. *Free Radic Biol Med* 1998; 24: 789-97.
- [50] Mendiratta S, Qu ZC, May JM. Enzyme-dependent ascorbate recycling in human erythrocytes: role of thioredoxin reductase. *Free Radic Biol Med* 1998; 25: 221-8.
- [51] Bocci V. Does ozone therapy normalize the cellular redox balance? Implications for therapy of human immunodeficiency virus infection and several other diseases. *Med Hypotheses* 1996; 46: 150-4.
- [52] Bocci V. Ozone as a bioregulator. *Pharmacology and toxicology of ozonotherapy today. J Biol Regul Homeost Agents* 1996; 10: 31-53.
- [53] Petersen DR, Doorn JA. Reactions of 4-hydroxynonenal with proteins and cellular targets. *Free Radic Biol Med* 2004; 37: 937-45.
- [54] Siems W, Grune T. Intracellular metabolism of 4-hydroxynonenal. *Mol Aspects Med* 2003; 24: 167-75.
- [55] Awasthi YC, Ansari GA, Awasthi S. Regulation of 4-hydroxynonenal mediated signaling by glutathione S-transferase. *Methods Enzymol* 2005; 401: 379-407.
- [56] Alary J, Geuraud F, Cravedi JP. Fate of 4-hydroxynonenal *in vivo*: disposition and metabolic pathways. *Mol Aspects Med* 2003; 24: 177-87.
- [57] Dianzani MU. 4-Hydroxynonenal and cell signalling. *Free Radic Res* 1998; 28: 553-60.
- [58] Claiborne A, Yeh JI, Mallett TC, *et al.* Protein-sulfenic acids: diverse roles for an unlikely player in enzyme catalysis and redox regulation. *Biochemistry* 1999; 38: 15407-16.
- [59] Bocci V, Aldinucci C, Mosci F, Carraro F, Valacchi G. Ozonation of human blood induces a remarkable upregulation of heme oxygenase-1 and heat stress protein-70. *Mediators Inflamm* 2007; 2007: 26785.
- [60] Pittler MH, Armstrong NC, Cox A, Collier PM, Hart A, Ernst E. Randomized, double-blind, placebo-controlled trial of autologous blood therapy for atopic dermatitis. *Br J Derm* 2003; 148: 307-13.
- [61] Torre-Amione G, Anker SD, Bourge RC, *et al.* Results of a non-specific immunomodulation therapy in chronic heart failure (ACCLAIM trial): a placebo-controlled randomised trial. *Lancet* 2008; 371: 228-36.

- [62] Sliwa K, Ansari AA. Immunosuppression as therapy for congestive heart failure. *Lancet* 2008; 371: 184-6.
- [63] Fildes JE, Shaw SM, Yonan N, Williams SG. Non-specific immunomodulation in chronic heart failure. *Lancet* 2008; 37: 2083.
- [64] Bocci V. The failure of the ACCLAIM trial is due to an irrational technology. *Int J Cardiol* 2008, in press. DOI:10.1016/j.ijcard.2008.10.001.
- [65] Bocci V, Di Paolo N, Garosi G, et al. Ozonation of blood during extracorporeal circulation. I. Rationale, methodology and preliminary studies. *Int J Artif Organs* 1999; 22: 645-51.
- [66] Travagli V, Zanardi I, Gabbriellini A, Paccagnini E, Bocci V. Are dialysis devices usable as ozone gas exchangers? *Artif Organs* 2009, in press. DOI:10.1111/j.1525-1594.2009.00767.x.
- [67] Bocci V, Zanardi I, Travagli V, Di Paolo N. Oxygenation-ozonation of blood during extracorporeal circulation: *in vitro* efficiency of a new gas exchange device. *Artif Organs* 2007; 31: 743-8.
- [68] Bocci V, Travagli V, Zanardi I, Di Paolo N, Tongs C. Treatment of human blood during extracorporeal circulation using a gas exchange device (GED) in patients. US Patent 60/977,149, October 13th 2007.
- [69] Di Paolo N, Bocci V, Salvo DP, et al. Extracorporeal blood oxygenation and ozonation (EBOO): a controlled trial in patients with peripheral artery disease. *Int J Artif Organs* 2005; 28: 1039-50.
- [70] Wenzel RP, Edmond MB. The evolving technology of venous access. *N Engl J Med* 1999; 340: 48-50.
- [71] Bergqvist D. Salvage of critically ischaemic limbs. *Lancet* 1999; 354: 1920-1.
- [72] Lu XW, Idu MM, Ubbink DT, Legemate DA. Meta-analysis of the clinical effectiveness of venous arterialization for salvage of critically ischaemic limbs. *Eur J Vasc Endovasc Surg* 2006; 31: 493-9.
- [73] Kang N, Jackson WA, Dasgupta PK, Anderson TA. Perchlorate production by ozone oxidation of chloride in aqueous and dry systems. *Sci Total Environ* 2008; 405: 301-9.
- [74] Truong GL, De Laat J, Legube B. Effects of chloride and sulfate on the rate of oxidation of ferrous ion by H₂O₂. *Water Res* 2004; 38: 2383-93.
- [75] Goldmann BU, Rudolph V, Rudolph TK, et al. Neutrophil activation precedes myocardial injury in patients with acute myocardial infarction. *Free Radic Biol Med* 2009, in press. DOI:10.1016/j.freeradbiomed.2009.04.004.
- [76] Ikonomidis S, Tsaousis P, Fyntanis A, Iliakis EM. New data regarding the use of oxidative stress (ozone therapy) in the former Soviet Union Countries. *Rivista Italiana di Ossigeno-Ozonoterapia* 2005; 4: 40-3.
- [77] Bocci V, Aldinucci C, Bianchi L. The use of hydrogen peroxide as a medical drug *Rivista Italiana di Ossigeno-Ozonoterapia* 2005; 4: 30-9.
- [78] Barco G. Un caso di linfoma non-Hodgkin non responsivo alle comuni terapie e trattato con ossigeno poliatomico liquido (OPL) somministrato in continuo per *via* venosa centrale e temozolomide. *Int J Ozone Ther* 2007; 6: 157-62.
- [79] Cacace F, De Petris G, Troiani A. Experimental detection of tetraoxygen. *Angew Chem Int Ed Engl* 2001; 40: 4062-5.
- [80] Ball P. New form of oxygen found. *Nat News* 2001; DOI:10.1038/news011122-3.
- [81] Matejcik S, Cicman P, Kiendler A, et al. Low-energy electron attachment to mixed ozone/oxygen clusters. *Chem Phys Lett* 1999; 261: 437-42.
- [82] Murai A, Nakajima T, Tahara N. Verification of ozone clusters (O₃ & O₉). *Ozone: Sci Eng* 2003; 25: 211-21.
- [83] Viebahn-Hänsler R. Allgemeine Eigenschaften des Ozons. In: *Viebahn-Hänsler R, Knoch HG Eds, Ozon-Handbuch. Grundlagen. Prävention. Therapie. Kaufbeuren, Ecomed Verlagsgesellschaft* 2001; II-1.1: 1-14.
- [84] Belianin II, Abdullaev RIU. Use of soluble ozone in combined treatment of pulmonary tuberculosis: lipid peroxidation and blood antioxidative defense systems. *Probl Tuberk* 2000; 3: 41-4.
- [85] Michaeli D, Michaeli M. Improved radial expansible retractor for minimally invasive surgery. PCT application number WO 2007/069232.
- [86] Travagli V, Zanardi I, Bocci V. Topical applications of ozone and ozonated oils as anti-infective agents: an insight into the patent claims. *Recent Pat Anti Infect Drug Discov* 2009, in press.
- [87] Sweet F, Kao MS, Lee SC, Hagar WL, Sweet WE. Ozone selectively inhibits growth of human cancer cells. *Science* 1980; 209: 931-3.
- [88] Pelloski CE, Ballman KV, Furth AF, et al. Epidermal growth factor receptor variant III status defines clinically distinct subtypes of glioblastoma. *J Clin Oncol* 2007; 25: 2288-94.
- [89] Sampson JH, Archer GE, Mitchell DA, Heimberger AB, Bigner DD. Tumor-specific immunotherapy targeting the EGFRvIII mutation in patients with malignant glioma. *Semin Immunol* 2008; 20: 267-75.
- [90] Lynch E. Evidence-based efficacy of ozone for root canal irrigation. *J Esthet Restor Dent* 2008; 20: 287-93.
- [91] Loncar B, Stipetic MM, Matosevic D, Tarle Z. Ozone application in dentistry. *Arch Med Res* 2009; 40: 136-7.
- [92] Bocci V, Borrelli E, Valacchi G, Luzzi E. Quasi-total-body exposure to an oxygen-ozone mixture in a sauna cabin. *Eur J Appl Physiol Occup Physiol* 1999; 80: 549-54.
- [93] He QC, Tavakkol A, Wietecha K, Begum-Gafur R, Ansari SA, Polefka T. Effects of environmentally realistic levels of ozone on stratum corneum function. *Int J Cosmet Sci* 2006; 28: 349-57.
- [94] Bocci V. Quasi-total body exposure to oxygen-ozone. In: *Ozone. A new medical drug. Dordrecht, The Netherlands, Springer* 2005; 56-65.
- [95] Bocci V. The paradoxical effect of ozone in orthopaedic diseases. The problem of back-ache. In: *Ozone. A new medical drug. Dordrecht, The Netherlands, Springer* 2005; 198-208.
- [96] Kakigi R, Inui K, Tamura Y. Electrophysiological studies on human pain perception. *Clin Neurophysiol* 2005; 116: 743-63.
- [97] Gallucci M, Puglielli E, Splendiani A, Pistoia F, Spacca G. Degenerative disorders of the spine. *Eur Radiol* 2005; 15: 591-8.
- [98] Rathmell JP. A 50-year-old man with chronic low back pain. *JAMA* 2008; 299: 2066-77.
- [99] Samanta A, Beardsley J. Low back pain: which is the best way forward. *BMJ* 1999; 318: 1122-3.
- [100] McCord JM. Free radicals and inflammation: protection of synovial fluid by superoxide dismutase. *Science* 1974; 185: 529-31.
- [101] Curran SF, Amoruso MA, Goldstein BD, Berg RA. Degradation of soluble collagen by ozone or hydroxyl radicals. *FEBS Lett* 1984; 176: 155-60.
- [102] Ueno I, Hoshino M, Miura T, Shinriki N. Ozone exposure generates free radicals in the blood samples *in vitro*. Detection by the ESR spin-trapping technique. *Free Radic Res* 1998; 29: 127-35.
- [103] Bocci V, Pogni R, Corradeschi F, et al. Oxygen-ozone in orthopaedics: EPR detection of hydroxyl free radicals in ozone-treated "nucleus pulposus" material. *Riv Neuroradiol* 2001; 14: 55-9.
- [104] Halliwell B. Free radicals and antioxidants: a personal view. *Nutr Rev* 1994; 52: 253-65.
- [105] Alexandre A, Buric J, Corò L, Rigobello L, Scopetta S. Discolisi percutanea mediante O₂O₃ intradiscale. In *Proceedings: I Congresso IMOS, Italia, Siena, 2-4 novembre 2000*; pp.7-8.
- [106] Silver FH, Glasgold AI. Cartilage wound healing. An overview. *Otolaryngol Clin North Am* 1995; 28: 847-64; Trippel SB. Growth factor actions on articular cartilage. *J Rheumatol* 1995; 43: 129-32.
- [107] Borrelli E, Bocci V. Basic biological and therapeutic effects of ozone therapy in human medicine. In: *Munter R Ed, Ozone Science and Technology, in Encyclopedia of Life Support Systems. Oxford, Eolss Publisher* 2008.
- [108] Fabris G, Tommasini G, Petralia B, et al. L'ossigeno-ozono terapia intra-foraminale. *Riv Neuroradiol* 2001; 14: 61-6.
- [109] Alexandre A, Pentimalli L, Rigobello L, Corò N. In: *Ceccherelli F, Giron F, Eds, L'Ozonoterapia nel 2000. Torino, Edizioni Libreria Cortina* 2000; 141-4.
- [110] Pellicanò G, Martinelli F, Tavanti V, et al. The Italian Oxygen-ozone therapy Federation (FIO) study on oxygen-ozone treatment of herniated disc. *Intern J Ozone Ther* 2007; 6: 7-15.
- [111] Zambello A, Fara B, Tabaracci G, et al. Epidural steroid injection vs paravertebral O₂O₃ infiltration for symptomatic herniated disc refractory to conventional treatment. A prospective randomized study. *Riv It Ossigeno-Ozonoterapia* 2006; 5: 123-27.
- [112] Gallucci M, Limbucci N, Zugaro L, et al. Sciatica: treatment with intradiscal and intraforaminal injections of steroid and oxygen-ozone versus steroid only. *Radiology* 2007; 242: 907-13.
- [113] Schulz S, Häussler U, Mandic R, et al. Treatment with ozone/oxygen-pneumoperitoneum results in complete remission of rabbit squamous cell carcinomas. *Int J Cancer* 2008; 122: 2360-7.

*Mechanisms of Action and Chemical-Biological Interactions**Current Drug Therapy, 2009, Vol. 4, No. 3 173*

- [114] Bocci V. Does ozone really "cure" cancer? *Int J Cancer* 2008; 123: 1222.
- [115] Bocci V. Ozone therapy in cancer. In: *Ozone. A new medical drug*. Dordrecht, The Netherlands, Springer 2005; 162-175.
- [116] Bocci V. Rectal insufflation of oxygen-ozone. In: *Ozone. A new medical drug*. Dordrecht, The Netherlands, Springer 2005; 49-56.
- [117] Bocci V, Borrelli E, Corradeschi F, Valacchi G. Systemic effects after colorectal insufflation of oxygen-ozone in rabbits. *Int J Med Biol Environ* 2000; 28: 109-13.
- [118] Kulikov AG, Turova EA, Shcherbina TM, Kisileva OM. Efficacy of different methods of ozone therapy in vascular complication of diabetes mellitus. *Vopr Kurortol Fizioter Lech Fiz Kult* 2002; 5: 17-20.
- [119] Hernández Rosales FA, Calunga Fernández JL, Turrent Figueras J, Menéndez Cepero S, Montenegro Perdomo A. Ozone therapy effects on biomarkers and lung function in asthma. *Arch Med Res* 2005; 36: 549-54.
- [120] Martínez-Sánchez G, Al-Dalain SM, Menéndez S, *et al*. Therapeutic efficacy of ozone in patients with diabetic foot. *Eur J Pharmacol* 2005; 523: 151-61.
- [121] León Fernández OS, Ajamieh HH, Berlanga J, *et al*. Ozone oxidative preconditioning is mediated by A₁ adenosine receptors in a rat model of liver ischemia/reperfusion. *Transpl Int* 2008; 21: 39-48.

Received: April 20, 2009

Revised: May 22, 2009

Accepted: May 26, 2009

GModel

ATH-11425; No. of Pages 2

ARTICLE IN PRESS

Atherosclerosis xxx (2010) xxx–xxx



Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Invited commentary

The irrationality of a non-specific immunomodulation therapy used in cardiovascular diseases deserves a critical comment

Velio Bocci^{a,*}, Iacopo Zanardi^b, Valter Travagli^b^a Department of Physiology, University of Siena, Viale Aldo Moro 2, 53100 Siena, Italy^b Department of Pharmaceutical Chemistry and Technology, University of Siena, Viale Aldo Moro 2, 53100 Siena, Italy

ARTICLE INFO

Article history:

Received 3 February 2010

Received in revised form 9 April 2010

Accepted 13 April 2010

Available online xxx

Reprinted with permission from Elsevier Ireland Ltd. © 2010
doi:10.1016/j.atherosclerosis.2010.04.014

In 1997, Bulmer et al. [1] proposed a new procedure consisting in placing 10 mL of anticoagulated (+2 mL sodium citrate) of the patient's venous blood in a VC7000A system (Celacade™, Vasogen Inc., Mississauga, ON, Canada) where it was exposed to an oxygen/ozone gas mixture (ozone concentration 15.35 g/m³) delivered into the blood at a flow of 240 mL/min and UV light (253.7 nm) at a temperature of 42.5 °C for about 20 min. The treated blood sample was removed from the system and immediately administered by intragluteal injection to the donor patient. Two treatments were given on consecutive days, followed by a third on day 14. Subsequent treatments were given at 4-week (28 days) intervals for at least 22 weeks, for a total of 8 injections.

The procedure uses an expensive device able to deliver an enormously toxic dose of ozone (107.5 mg per mL of blood) plus an undetermined UV irradiation at 42.5 °C. The final ozone dose is about 15,000-fold higher than the average ozone dose used during the classical ozonated autohemotherapy [2] and the extremely high oxidation of blood causes a complete denaturation of blood components [3]. This procedure was invented aiming to establish a non-specific immunomodulation therapy (IMT) in the hope of reducing the inflammatory process and the chronic oxidative stress present in vascular disease. It has proved to be useless in an AIDS trial [4] and in a multicenter, randomized, double-blind, placebo-controlled study in 533 patients with symptomatic peripheral arterial disease (PAD), called the SIMPADICO trial [5]. It is most important noting that this trial had to be stopped 3 months early because it did not show any improvement in PAD and caused a significantly higher rate of malignancies in the IMT group [6]. Although in a pilot study [7] of 73 patients with heart failure the IMT seemed to result in a reduction of mortality, a subsequent

multicenter study in 2426 patients [8], called the ACCLAIM trial, in chronic heart failure resulted in a "disappointing" results. In this trial no particular cancer predominated "although an imbalance was seen in reports of colorectal cancer (nine patients in the IMT group and three in the placebo group). The proponents of IMT [1,9] support the concept that after the IM administration of heavily denatured blood, an immune modulation ensues with an up-regulation in the production of the anti-inflammatory cytokines such as IL-10 and TGF-β and inhibition of proinflammatory cytokines, such as TNF-α, IL-1 and IL-6. As chronic heart failure and limb ischemia are affections linked to inflammation and chronic oxidative stress, in theory the reduction of proinflammatory cytokines may down-regulate chronic inflammation and delay the progress of the disease. However, the previous failure of the SIMPADICO trial not only was a premonition of poor result in chronic heart failure but also suggested that a strong immune suppression may favour neoplastic growth. Sliwa and Ansari [10] pointed out several potential problems but did not comment the wrong oxidative technology. An intermediate position of wait and see has been concurrently adopted by Sporter et al. [11]. On the other hand, one of us [12,13] made specific criticism regarding the irrational technology and deeply dissented with Vasogen's (since October 2009 merged in IntelliPharmaceutics International Inc.) hypothesis. After two decades of studying the mechanisms of action in blood, reviewed in [2], the therapeutic range of ozone as a medical drug (0.21–1.68 mmol/mL of ozone per mL of anticoagulated blood) has been defined. Ozone is a most reactive gas and inherently toxic but, if judiciously used, it is very useful in vasculopathies because it enhances vasodilation, it increases the delivery of both oxygen and growth factors in ischemic tissues and it does up-regulate several antioxidant enzymes and above all of heme-oxygenase-1 [14]. Immunomodulation may be only a small additional factor. The misinterpretation of the real mechanisms of action and the obstinate use of a wrong approach can explain the

* Corresponding author. Tel.: +39 0577234226; fax: +39 0577234219.
E-mail address: bocci@unisi.it (V. Bocci).

G Model

ATH-11425; No. of Pages 2

2

ARTICLE IN PRESS

V. Bocci et al. / *Atherosclerosis xxx (2010) xxx–xxx*

“disappointing” results and the previous failure in treating chronic limb ischemia in the SIMPADICO trial [5,6]. Moreover a randomized clinical trial has proved the validity and safety of ozone therapy in severe chronic limb ischemia [15]. For these reasons, believing that the procedure had been definitively entombed, we were surprised to read a recent paper by Marfella et al. [16] claiming that IMT may improve wound healing and limb salvage in patients with chronic limb ischemia. Although the same technology had been used, they obtained positive results absent in the SIMPADICO trial that was not cited in their paper. They also used a masked saline placebo in the control group where it would have been more appropriate to re-inject the untreated autologous blood. The reason of these controversial results remains unknown, unless there is a different reactivity between American and Neapolitan patients. One reasonable hypothesis may regard a benevolent and unknown deficiency of the VC7000A system to deliver the excess of ozone. Moreover the authors, likely unaware that this immunosuppressive therapy may enhance tumorigenesis, have not checked this critical part and it should be suggested the need of exploring this aspect in their patients.

The reason of our concern is that the planned commercialization of such a device ought to be prohibited because practitioners may use this method in vascular patients unaware of the poor medical benefit and the risk of enhancing neoplastic growth.

Conflict of interest

The authors declare that they have no conflict of interest.

References

- [1] Bulmer J, Bolton AE, Pockley AG. Effect of combined heat, ozonation and ultraviolet irradiation (VasoCare) on heat shock protein expression by peripheral blood leukocyte populations. *J Biol Regul Homeost Agents* 1997;11:104–10.
- [2] Bocci V, Borrelli E, Travagli V, Zanardi I. The ozone paradox: ozone is a strong oxidant as well as a medical drug. *Med Res Rev* 2009;29:646–82.
- [3] Travagli V, Zanardi I, Bernini P, et al. Effects of ozone blood treatment on the metabolite profile of human blood. *Int J Toxicol* 2010;29:165–74.
- [4] Garber GE, Cameron DW, Hawley-Foss N, Greenway D, Shannon ME. The use of ozone-treated blood in the therapy of HIV infection and immune disease: a pilot study of safety and efficacy. *AIDS* 1991;5:981–4.
- [5] SIMPADICO—Study of Immune Modulation Therapy in Peripheral Arterial Disease and Intermittent Claudication Outcomes. Available at: <http://clinicaltrials.gov/ct2/show/NCT00111826> [accessed April 9, 2010].
- [6] Olin JW. Peripheral arterial disease: efficacy of immune modulation. In: Presented at: 55th Annual Scientific Sessions, Smaller Late-Breaking Clinical Trials I. 2006. Available at: <http://incirculation.net/3430.68539.aspx?parentaid=67695> [accessed April 9, 2010].
- [7] Torre-Amione G, Sestier F, Radovancevic B, Young J. Effects of a novel immune modulation therapy in patients with advanced chronic heart failure: results of a randomized, controlled, phase II trial. *J Am Coll Cardiol* 2004;44:1181–6.
- [8] Torre-Amione G, Anker SD, Bourge RC, et al. Results of a non-specific immunomodulation therapy in chronic heart failure (ACCLAIM trial): a placebo-controlled randomised trial. *Lancet* 2008;371:228–36.
- [9] Bolton AE. Biologic effects and basic science of a novel immune-modulation therapy. *Am J Cardiol* 2005;95:24C–9C.
- [10] Sliwa K, Ansari AA. Immunosuppression as therapy for congestive heart failure. *Lancet* 2008;371:184–6.
- [11] Sporter RJ, Kim JH, Frishman WH. Device-based nonspecific immunomodulation therapy (Celacade), and its potential role in the treatment of chronic heart failure. *Cardiol Rev* 2008;16:280–7 [Erratum in: *Cardiol Rev* 2009;17:43].
- [12] Bocci V. Non-specific immunomodulation in chronic heart failure. *Lancet* 2008;371:2083.
- [13] Bocci V. The failure of the ACCLAIM trial is due to an irrational technology. *Int J Cardiol* 2010;139:304–5.
- [14] Bocci V, Aldinucci C, Mosci F, et al. Ozonation of human blood induces a remarkable upregulation of heme oxygenase-1 and heat stress protein-70. *Mediators Inflamm* 2007;26785, doi:10.1155/2007/26785.
- [15] Di Paolo N, Bocci V, Salvo DP, et al. Extracorporeal blood oxygenation and ozonation (EBOO): a controlled trial in patients with peripheral artery disease. *Int J Artif Organs* 2005;28:1039–50.
- [16] Marfella R, Luongo C, Coppola A, et al. Use of a non-specific immunomodulation therapy as a therapeutic vasculogenesis strategy in no-option critical limb ischemia patients. *Atherosclerosis* 2010;208:473–9.

Please cite this article in press as: Bocci V, et al. The irrationality of a non-specific immunomodulation therapy used in cardiovascular diseases deserves a critical comment. *Atherosclerosis* (2010), doi:10.1016/j.atherosclerosis.2010.04.014



Are Dialysis Devices Usable as Ozone Gas Exchangers?

**Valter Travagli, *Iacopo Zanardi,
*Alessandro Gabbrielli, †Eugenio Paccagnini,
and ‡Velio Bocci
*Dipartimento Farmaco Chimico Tecnologico;
†Dipartimento di Biologia Evolutiva; and
‡Dipartimento di Fisiologia, Università degli Studi di
Siena, Siena, Italy*

Abstract: A study aimed to compare the efficiency of the ozone transfer of four hydrophilic dialysis filters, and one hydrophobic gas-exchange device (GED) has been performed. Obviously, the former should be specifically used only for dialysis. Unfortunately, some clinicians incautiously use them as GEDs. It has been shown that: (i) dialysis filters present a wide range of gas-exchange yield (from 0 up to 70%), often related to variability according to the treatment time; (ii) by scanning microscopy, it has been noticed that hollow fibers are somewhat altered by ozone; and (iii) because their constitutive materials may not be ozone-resistant, they may release toxic compounds harmful for the patients. On the contrary, the appropriate GED is ozone-transfer efficient, is ozone-resistant, and is suitable for blood autotransfusion and ozonation. **Key Words:** Ozone—Extracorporeal circulation—Hollow fibers—Gas-exchange device.

To the best of our knowledge, the first approach aiming to simultaneously oxygenate and ozonate human blood was proposed by Wainwright (1). In such a case, blood withdrawn from a patient runs inside a tilted glass tube against a gas mixture composed of oxygen-ozone flowing in a countercurrent direction. The procedure appeared to be effective, but it presented a risk of viral infections even though the system had to be sterilized for each treatment. A similar stainless steel system recently proposed by Latino and Keyser (2) has the same drawbacks. Since 1992, in cooperation with the Nephrology Unit of the Polyclinic of Siena (Italy), a dialysis-like system, where the dialysate was substituted with O₂-O₃ gaseous mixture similarly directed in a countercurrent fashion for ozonating blood, was investigated. The dialysis filter is referred to as an artificial kidney. Blood withdrawn from the patient runs inside hollow fibers of different hydrophilic composition while the dialysate runs outside in countercurrent fashion. Dialysis membranes not only need to be efficient at clearing wastes, but must also be biocompatible with

doi:10.1111/j.1525-1594.2009.00767.x

Received August 2008; revised January 2009.

Address correspondence and reprint requests to Prof. Velio Bocci, Dipartimento di Fisiologia, Università degli Studi di Siena, Viale Aldo Moro 2, 53100 Siena, Italy. E-mail: bocci@unisi.it

Reprinted with permission from *Artif Organs*, Vol. 34, No.2 - © 2010

THOUGHTS AND PROGRESS

171

human blood. Filter permeability is influenced by pore size, number of pores, and thickness of the membrane. The surface area of the membrane determines the available area for diffusion and ultrafiltration (3). After a few years of research, it was realized that the system was unsuitable because the dialysis filters: (i) were poorly effective for gas exchange; (ii) may have reacted with ozone, possibly releasing toxic materials into the blood; and (iii) caused a loss of solutes and water. Moreover, they allowed some platelet adhesion, leukocyte activation, and produced some immune responses (4,5). The conclusion that dialysis filters were unsuitable for blood ozonation, chiefly for the hydrophilic nature of the hollow fibers and the possible release of toxic compounds, has been reached. For this reason, the examination of single-use and ozone-resistant hydrophobic membranes, made of polypropylene, hence impermeable to water but freely permeable to O_2 - O_3 , was started. At first, the gas-exchange devices (GEDs), even using heparin, were minimally biocompatible (6) until 2 years ago, when a suitable phosphorylcholine-coated one was used (7). In the latest GED, denominated D100, in comparison to dialysis filters, blood runs outside the fibers against the gas mixture inside. This GED has proved to be quite effective and atoxic when used for blood ozonation (8,9). Common dialysis filters are usually sterilized with gamma radiation, even if both ethylene oxide and steam could be used (10). However, ozone is also occasionally used for the same purpose (11–16), as well as for polymer grafting (17–19) or, unfortunately, for performing recirculatory hemoperfusion against O_2 - O_3 , implying a risk for the patients (20).

The aim of this report is to show that, during these unorthodox practices using dialysis filters, their *in vitro* gas-exchange efficiency is often minimal and, even if such is not the case, they might potentially be hazardous for the patient because they are not specifically tested and/or approved for such purposes.

MATERIALS AND METHODS

Hollow fiber modules

The following four dialysis filters have been used and codified as follows:

- 1 Heparinized cuprophane, Cobe Centrysystem 550, Secon GmbH, Germany;
- 2 Cellulose triacetate high efficiency hollow fiber, CT150G, Baxter S.A., France;
- 3 Polysulfone Rexbrane, Rexeed-18L, Asahi, Japan; and

- 4 Polymethylmethacrylate, Filtryzer B3-2.0A, Toray, Japan.

In contraposition, a new and suitable GED, namely D100, made of phosphorylcholine-coated polypropylene (Sorin, Mirandola, Italy) was used as reference standard. All the devices and ancillary materials were sterile and used only once.

Schematic diagram of the circuit and operative conditions

Figure 1 describes in detail the various operative conditions and embodiments.

The dialysis filters, the GED, and lines were routinely rinsed with 2-L saline before starting the perfusion simulated by saline phosphate buffer (pH 7.4), in the presence of KI (0.12 M). The various ozone concentrations used throughout the experiments (between 0.5 and 2.5 $\mu\text{g}/\text{mL}$) were generated by Ozonline ECO₃ (Torino, Italy) using only pure, medical-grade oxygen representing at least 95% of the gas mixture at a feed flow of 15 L/h. Ozone concentration was continuously monitored by a photometer, periodically checked by iodometric titration. The overall time of ozone treatment was 60 min.

Transfer and total yield of ozone were evaluated both during the early phase of treatment and after 60 min of ozone flowing, simulating the time of a therapeutic session.

When ozone, flowing in a countercurrent fashion outside the hollow fibers, permeated these same, it immediately reacted with the KI solution, generating I_2 , which was titrated with $\text{Na}_2\text{S}_2\text{O}_3$ (0.0001–0.1 N) (21) (see Scheme 1).

Scheme 1. Iodine titration

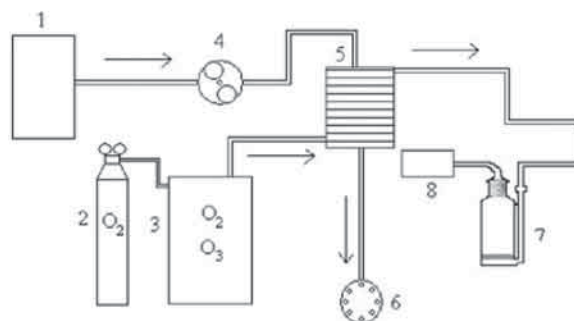
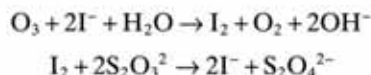


FIG. 1. Schematic diagram of the experimental device: 1 saline solution or buffer; 2 oxygen supply; 3 ozone generator with photometer; 4 peristaltic pump; 5 dialysis filter or GED; 6 sample collector; 7 silica gel trap; 8 ozone destructor. The peristaltic pump was always kept in "ON" position throughout the experiments.



The equation indicates the reaction elicited by ozone and the classical reducing reaction for measuring the ozone. Furthermore, blank tests under the same conditions using only oxygen for the same time have been carried out.

Scanning Electron Microscopy (SEM) characterization

The morphology of hollow fibers before and after ozone treatment, or after phosphate buffered saline (pH 7.4) with and without I₂ (0.1 N) perfusion, was investigated by SEM studies. Both the external and internal hollow fiber surfaces were examined. The material was mounted on aluminum stubs by carbon conductive glue and coated with 20 nm gold in a Balzer's MED 010 sputtering device. The samples were finally observed with a Philips XL20 scanning electron microscope operating at an accelerating voltage of 5–20 kV, according to the material sensitivity.

Statistical analysis

Results were expressed as the mean of at least three independent measurements (CV% < 2). One-way analyses of variance performing the Bonferroni posttest (Instat software, version 3.0 GraphPAD Software, Inc., San Diego, CA) were used for the statistical analysis of the results. Significance was defined as a *P* value less than 0.05 (**P* < 0.05; ***P* < 0.01; ****P* < 0.001).

RESULTS AND DISCUSSION

Efficiency evaluation

Owing to the fact that ozone is a strong oxidant to be used with great concern and with proper devices, a precise knowledge of the ozone effects on the devices during the overall treatment is essential. When ozone, flowing in a countercurrent fashion outside the hollow fibers of the dialysis filters, interacts with the KI solution flowing inside the fibers, it immediately generates iodine, and in such a way, the device acquires an amber color. Transfer and total yield of ozone were evaluated during the early phase of treatment. The same evaluation was also repeated after 60 min of a continuous ozone flowing, simulating the time of a therapeutic session. Figure 2 shows the amount of ozone transferred of the four dialysis filters at the beginning (top) and at the end (bottom) of the experiment, respectively.

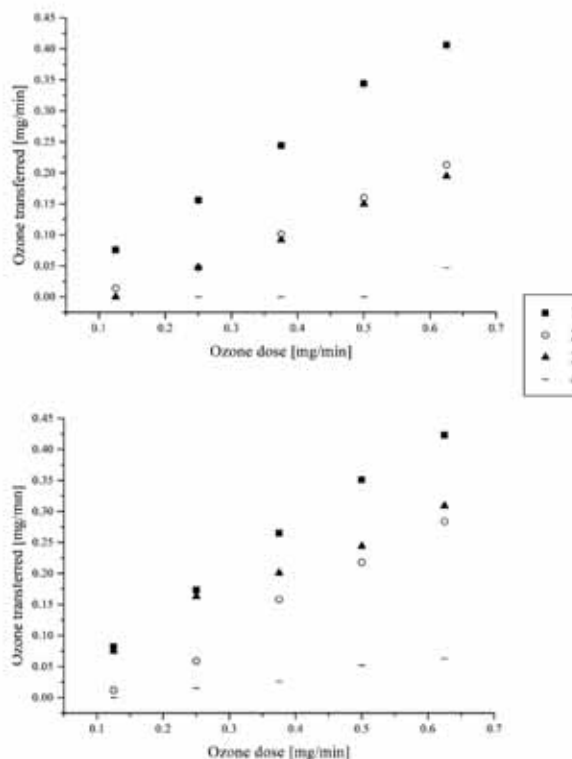


FIG. 2. Ozone transfer (mg/min) at the start (top) and at the end (bottom) of the ozone treatment. Numbers 1–4 refer to dialysis filters as described in "Materials and Methods."

Apart from filter 4, the ozone transfer increased in a linear fashion with respect to the ozone dose. Device 1 appears to be rather efficient and reproducible with time, followed by an almost analogous behavior of both devices 2 and 3, at least as far as the initial treatment is concerned. In fact, the presence of ozone inside the system 3 for about 60 min led to a significant variation in the ozone efficiency, especially for the doses up to 0.4 mg/min. On the contrary, device 4 seemed gas-transfer inefficient, even if a diffuse browning of the hollow fibers in proximity of the gas inlet and outlet was observed. Table 1 shows the ozone yield % at the beginning and at the end of the perfusion in relation to the ozone concentrations. In details it can be noted that while filter 4 is inefficient, filter 1 has yielded more constant and higher values. Moreover, filters 2 and 3 show a variable yield during the ozone treatment.

The efficiency and ozone yield achieved using the GED D100, where the KI solution flows outside the fibers, are shown in Fig. 3, in relation to the data at the start of the evaluation. The ozone transfer is almost quantitative and significantly higher than in dialysis filters (*P* < 0.001). No differences of values at

THOUGHTS AND PROGRESS

173

TABLE 1. Ozone yield of the four dialysis filters both at the start and at the end of experiments in relation to different ozone concentrations

Hollow fiber modules	Ozone yield [%]									
	0.5 µg/mL		1.0 µg/mL		1.5 µg/mL		2.0 µg/mL		2.5 µg/mL	
	Start	End	Start	End	Start	End	Start	End	Start	End
1	61	66**	62	69**	65	71**	69	70 n.s.	65	68*
2	11	10 n.s.	19	24**	27	42***	32	44***	34	45***
3	0	60***	19	65***	25	54***	30	49***	31	49***
4	0	0 n.s.	0	6***	0	7***	0	10***	8	10*

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

the end of the ozone treatment were observed, meaning a constant gas exchange (data not shown).

Owing to the fact that the gas mixture is composed of at least 95% medical oxygen, it must be pointed

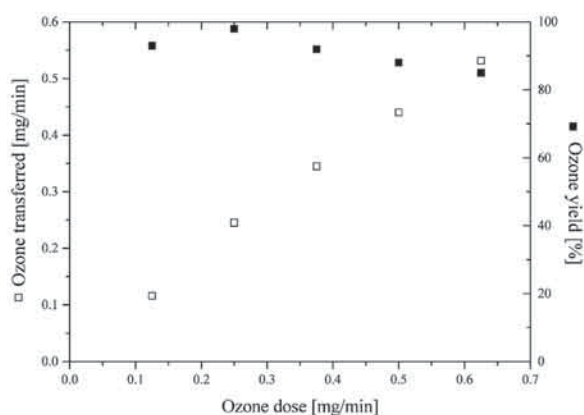
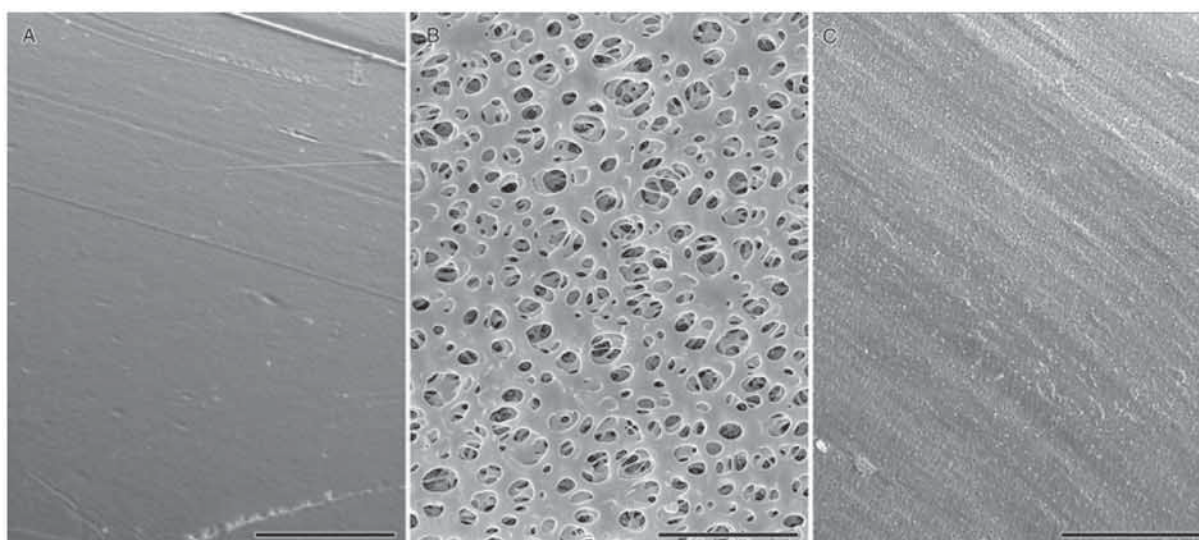
out that no appreciable oxidation of KI occurred during control experiments using only oxygen.

The morphology and structure of hollow fibers become the focus of the concern of the fiber integrity and how ozone significantly affects their stability during treatment.

SEM characterization

In order to examine whether ozone may damage the fiber membrane of the various filters, the hollow fiber surfaces were examined by SEM.

Hollow fiber specimens from filters 1, 2, and 4 were very sensitive to high-energy electrons and they were preferentially observed at 5 kV. Both the outer and the inner surface of the fibers above these three filters are smooth and continuous. Fibers from filters 1 and 4 are instead morphologically unaffected by the ozone flow (data not shown). In fibers from filter 2, the outer (Fig. 4A) and inner sides are unaltered by the ozone flow with respect to control (saline and KI solution), even though several little dips are always

**FIG. 3.** Ozone transfer (□) and ozone yield (■) for the GED.**FIG. 4.** Outer surface of the fibers from the devices after ozone treatment. (A) Filter 2. (B) Filter 3. (C) GED. Bar = 10 µm.

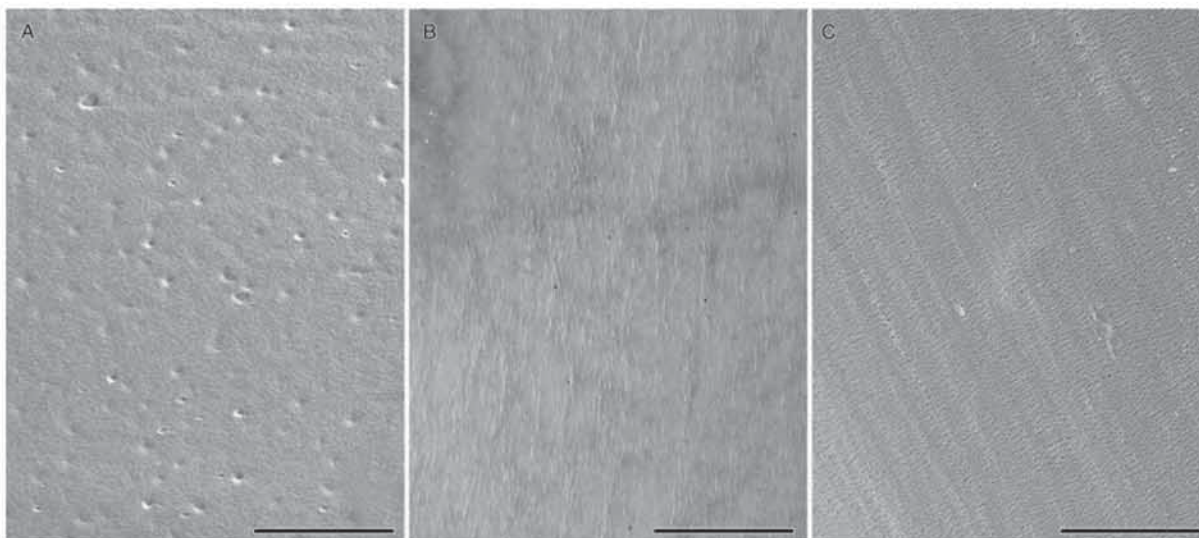


FIG. 5. Inner surface of the fibers from the devices after ozone treatment. (A) Filter 2. (B) Filter 3. (C) GED. Bar = 10 µm.

present in the inner side of the same fibers (Fig. 5A), and for this reason, no further ozone treatment evaluation is possible.

Specimens from filter 3 and from GED were observed at 10–20 kV. The outer surface of the filter 3 fibers (Fig. 4B) appears discontinuous as being made up of several stacked layers of a holey film. The inner side of this fiber, instead, has a barely wrinkled but continuous surface (Fig. 5B). In this side of the fibers and for the whole filter length, several structural alterations are observable as little dark spots, presumably due to the ozone flow (Fig. 6). This phenomenon suggests the reactivity of the fiber materials at the experimental conditions adopted. Both the outer (Fig. 4C)

and the inner (Fig. 5C) surface of the fibers from GED show the same morphological appearance, and they seem similar to each other. SEM observation at higher magnification of GED fibers reveals a very close reticular structure (Fig. 7), with longitudinal thread crossed with irregular and circularly arranged matter. The fibers from this filter appear morphologically unaltered by ozone treatment.

CONCLUSIONS

Dialysis filters, to a different extent, allow a non-constant transfer with time of the gas mixture O_2-O_3 into the saline–KI solution, variable in a proportional

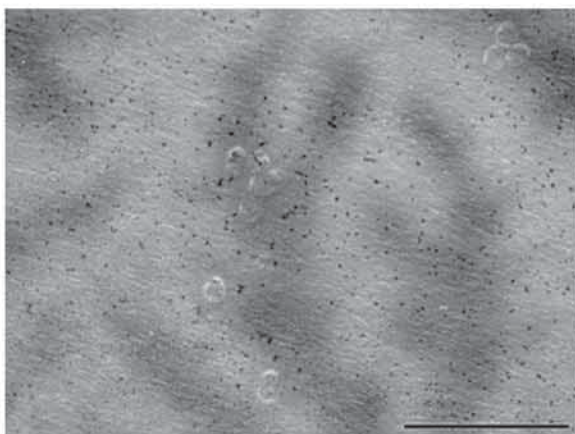


FIG. 6. Inner surface of fibers from filter 3. Many little dark spots are observable after ozone treatment. Bar = 5 µm.

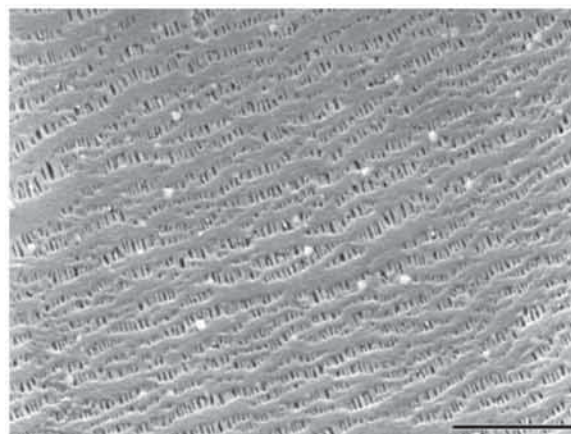


FIG. 7. The inner surface of the fibers from GED filter shows a very close reticular structure. Bar = 2 µm.

manner to the ozone amount for filters 2–4. Filter 1 allows an ozone exchange ranging from 61 to 69% at the initial stage of the experiment, until a 71% ozone exchange at the end is reached. Clearly, these filters are suitable for dialysis but cannot be used as gas exchangers, even if a quantitative passage of ozone is reached. In fact, dialysis filters are made of hydrophilic materials that are not ozone-resistant and therefore they may react with ozone, and they may release unwanted chemicals potentially harmful for the patient. Moreover, the GED is made of hydrophobic, ozone-resistant polypropylene-based fibers. On the basis of the present findings, it is hoped that anyone incautiously using dialysis filters for blood extravascular ozonation should correctly use the appropriate device. Eventually, considering the strong reactivity of ozone, its occasional use for sterilizing dialysis filters could be carefully evaluated because it may impair their function.

Acknowledgments: The authors are grateful to the Department of Nephrology, University of Siena, for providing the dialysis devices. The linguistic revision by Mrs. H. Carter is also gratefully acknowledged.

REFERENCES

1. Wainwright BA. Apparatus and method for inactivation of human immunodeficiency virus. *US Patent* 6,027,688, May 2, 1994.
2. Latino JS, Keyser SA. Viral inactivation using ozone. *US Patent Application* 20050189302 A1, October 11, 2004.
3. Pierratos A. New approaches to hemodialysis. *Ann Rev Med* 2004;55:179–89.
4. Bocci V, Di Paolo N, Garosi G, et al. Ozonation of blood during extracorporeal circulation. I: rationale, methodology and preliminary studies. *Int J Artif Organs* 1999;22:645–51.
5. Bocci V, Di Paolo N, Borrelli E, Larini A, Cappelletti F. Ozonation of blood during extracorporeal circulation II. Comparative analysis of several oxygenators-ozonators and selection of one type. *Int J Artif Organs* 2001;24:890–7.
6. Bocci V, Di Paolo N. Oxygenation-ozonation of blood during extracorporeal circulation (EBOO). Part III: a new medical approach. *Ozone Sci Eng* 2004;26:195–205.
7. Bocci V, Zanardi I, Travagli V, DiPaolo N. Oxygenation-ozonation of blood during extracorporeal circulation: in vitro efficiency of a new gas exchange device. *Artif Organs* 2007;31:743–8.
8. Di Paolo N, Bocci V, Gaggiotti E. Ozone therapy. *Int J Artif Organs* 2004;27:168–75.
9. Bocci V, Travagli V, Zanardi I, Di Paolo N, Tongs C. Treatment of human blood during extracorporeal circulation using a gas exchange device (GED) in patients. *US Patent Pending* n° 60/977,149, October 13, 2007.
10. Aucella F, Vigilante M, Gatta G, et al. Effects of ethylene oxide and steam sterilization on dialysis-induced cytokine release by cuprophan membrane. *Artif Organs* 2002;26:543–5.
11. Gal G, Kiss E, Foldes J, Dombi A. Disinfection of regenerated dialyzer with ozone. *Int J Artif Organs* 1992;15:461–4.
12. Amato RL. Disinfection of an RO system: clearing the issues. *Dial Transplant* 1995;24:244–9.
13. Masuda T, Masuda Y. Method for cleaning a dialyzer hemodialysis system. *US Patent* 6,800,248, March 21, 2001.
14. Amato RL, Curtis J. The practical application of ozone in dialysis. *Nephrol News Issues* 2002;16:27–30.
15. Shintani H, Suzuki E, Sakurai M. Determination of compounds inhibiting bacterial growth in sterilized medical devices. *Chromatographia* 2003;58:193–9.
16. Heng S, Yeung KL, Djafer M, Schrotter J-C. A novel membrane reactor for ozone water treatment. *J Membr Sci* 2007;289:67–75.
17. Yang M-C, Lin W-C. The grafting of chitosan oligomer to polysulfone membrane via ozone-treatment and its effect on anti-bacterial activity. *J Polym Res* 2002;9:135–40.
18. Xu J, Yuan Y, Shan B, Shen J, Lin S. Ozone-induced grafting phosphorylcholine polymer onto silicone film grafting 2-methacryloyloxyethyl phosphorylcholine onto silicone film to improve hemocompatibility. *Colloids Surf B Biointerfaces* 2003;30:215–23.
19. Kung F-C, Yang M-C. The effect of covalently bonded conjugated linoleic acid on the reduction of oxidative stress and blood coagulation for polysulfone hemodialyzer membrane. *Int J Biol Macromol* 2006;38:157–64.
20. Diang L-K. Ozone hemodiafiltration device. *US Patent Application* 20030073945 A1, October 17, 2001.
21. Masschelein W. Jodometric method for the determination of ozone in a process gas. In: Viebahn-Hänsler R, Knoch HG, eds. *Ozon-Handbuch. Grundlagen.Prävention.Therapie*, vol. IX-8. Landsberg: Ecomed, 2001;1–3.

Lumbar Vertebral Disc Pathology. New Frontiers: Ozone + Stem Cells

L. BRINA¹, A.G. POPANDOPULO²

¹ Orthopaedics 2[^] San Paolo Bari, Italy

² Institute of Urgent Recovery Surgery N.A. Gusak of AMS of Ukraine

Key words: lumbar vertebral disc, ozone, stem cells

ABSTRACT - *The authors show the results obtained in the treatment of degenerative lumbar disc pathology using paravertebral infiltrations of an oxygen-ozone mixture, in conjunction with autologous stem cell treatment, the latter isolated from bone marrow taken from the iliac crest of the same patient and cultured in the laboratory. The results, evaluated clinically and with MRI, before and after treatment, show an improvement in the trophism of the disc.*

Introduction

The treatment of disc herniation, or degenerative disc disease with paravertebral infiltration of oxygen-ozone mixture has already shown its advantages, as highlighted by other authors (Alexander).

Surgery and other methods have shown over time to lead to recurrent disc herniations and problems with adherence such as periradicular scar tissue formation. These additional disc diseases, especially the more destabilizing and debilitating, subsequently require new surgical interventions which are more invasive and debilitating and have led to serious problems of instability of the spine.

Until recently it had not been considered that it could be possible to improve the trophism of the disc and reduce the volume of the hernia in the lumbar canal using ozone. The ozone treatment affects the blood perfusion of the nerve roots, the epidural inflammation, the metabolism of nerve tissues and the metabolism of the matrix.

The action of ozone in these diseases, now confirmed by both the clinical literature and by our own practice, consists of:

- Anti-inflammatory action;
- Breaking the chain of prostaglandins, the cause of pain;
- Neoangiogenesis in the perineural arterial circulation (Brina).

Therefore, the infiltration at paravertebral intramuscular level produces analgesia and muscle relaxation as well as having anti-inflammatory, hyperoxygenating effects.

Our study showed that the combination of oxygen-ozone with stem cells has not only had the effects mentioned above, but - as demonstrated by MRI scans performed at the end of the treatment - also resulted in a reduction in the mass of the hernia, and improvement in the state of the disc, as shown by an increase in its height and trophism of the annulus.

At "Biostem", the Donetsk Center for Biotechnology, directed by Prof. Andriy G. Popandopulo, the implantation of autologous stem cells has long been used in the treatment of in-ternistic diseases, cardiac diseases, in plastic and cosmetic surgery, in the treatment of severe burns with loss of substance, in stroke rehabilitation and in orthopaedics.

Particularly in orthopaedics stem cells have been taken from the patients own bone marrow and cultured in the laboratory, for the treatment of osteochondritis and osteonecrosis.

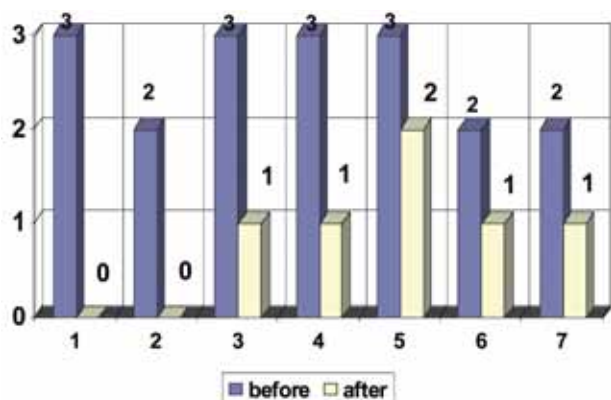
The purpose of our experiments, performed in co-operation between the orthopaedic department San Paolo di Bari Hospital and Biostem, was to study the effect of using a combined treatment of ozone-oxygen infiltration and autologous stem cell implantation to determine the regenerative capacity of the intervertebral discs.

Our study highlights the benefits of ozone treatments on degenerative disc diseases, but also takes this technique further and combines it with stem cell implantation.

It is crucial in this context to define the anatomy of the lumbar intervertebral disc, and its relationship with the vertebral body consisting of the cortical spongy-bone and cartilaginous plates.



Institute.



Scheme evidencing improvement of muscular contracture and lumbago.

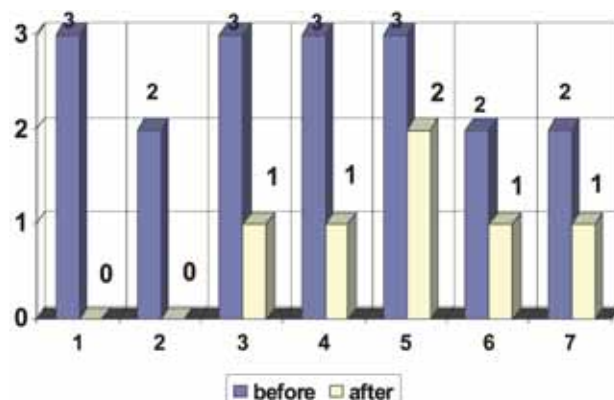


Chart showing improvement of sciatica symptomatology.

Disc:

- a) annulus fibrosus (fibrous-elastic in the peripheral region, cartilage on the inside);
- b) annulus consisting of concentric lamellae of fibrous tissue.

The vascularity of the disc is provided by blood vessels that enter and exit the disc through perforations in the central cartilaginous plate. These supplying arteries gradually atrophy in the first three decades of life, and subsequently nourishment is supplied solely by the lymphatic canals and by the circulation of extracellular fluid.

The Anatomy of the Diseased Disc

- difficulties in identifying the boundary between nucleus pulposus and inner part of the annulus fibrosus
- progressive loss of elasticity in the annulus fibrosus
- concentric injuries to the circumference of the periphery of annulus

- increase in size and extension of injury radiating both out towards the periphery of the disc, and inwards to the centre of the annulus (like the domino effect), opposite the posterior region (the area of the intervertebral disc placed under greatest strain by the load)
- atrophy of the blood vessels supplying the discs.

Ozone action in general:

- neoangiogenesis
- anti-inflammatory action
- relaxant action.

Action of ozone on intervertebral disc:

- angiogenesis
- neof ormation of proteins and fibrocytes in annulus

Techniques:

- intradiscal
- paravertebral
- small and large autohaemotransfusion



Biostem center, doctors at work.



Another particular of doctors at work.



Cellular sampling from the iliac crest.



Cellular multiplication cell.



Different phase of cellular sampling from the Hiliac crest.

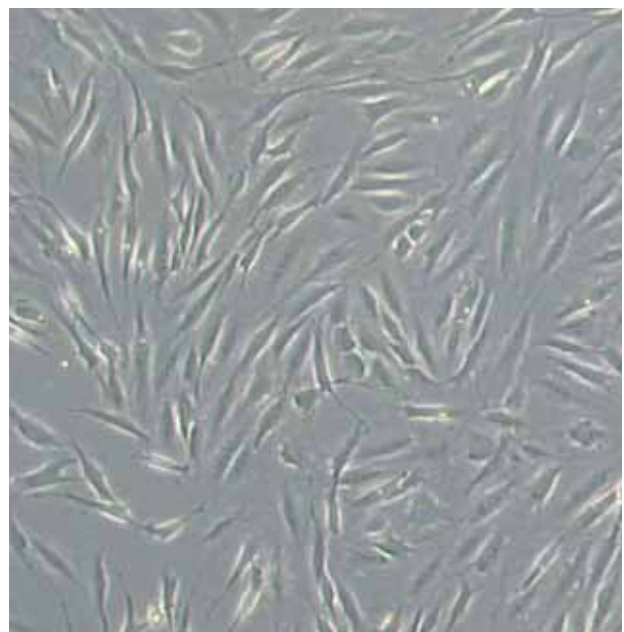
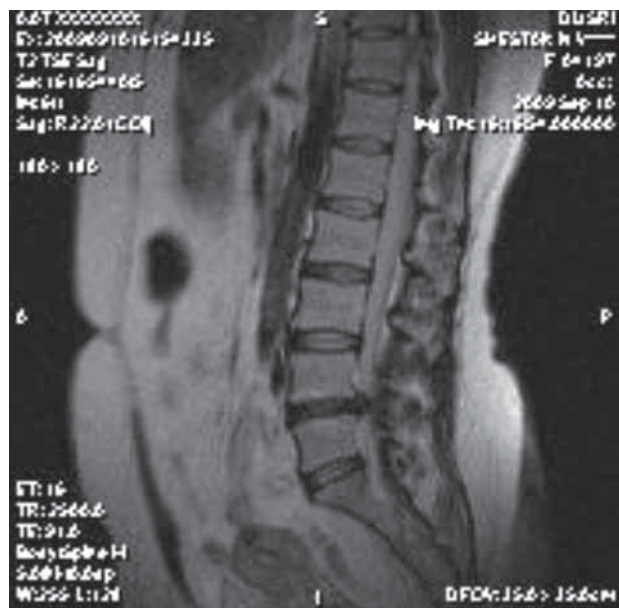
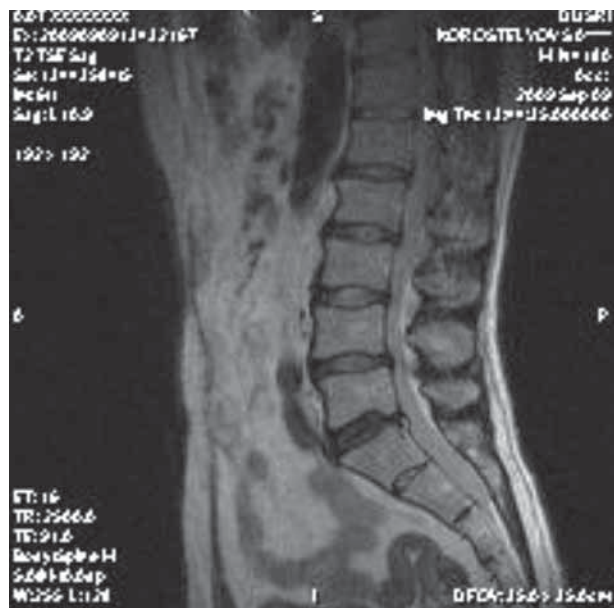


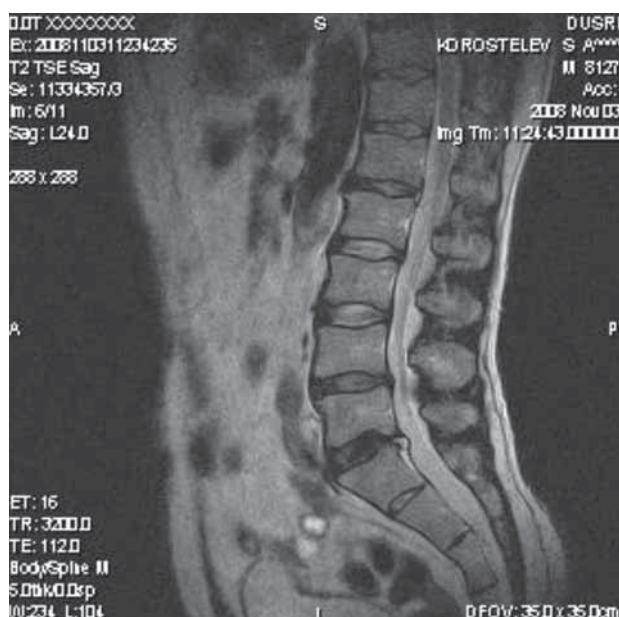
Image electron microscopy.



MRI of the lumbar spine before treatment.



MI after treatment reduction of herniated material and increase of disc height.



Different case.



Different case MRI after treatment.

Materials and methods

20 patients were treated: 17 males and 3 females, aged between 34 and 63. The average patient age was 42

The diseases treated, as shown in Table 1, were:

- osteocondritis of the disc
- disc protrusion
- bulging disc
- intraforaminal hernias.

Investigations performed prior to treatment were:

- Spinal MRI

X ray of lumbosacral spine in the upright (2 projections) + dynamic radiographs in maximal flexion and extension.

Paravertebral infiltration technique used

2 doses of 10 ml to the side to be treated, with 27G syringe and needle

Dosage 12-14 microgrammes/ ml of ozone.



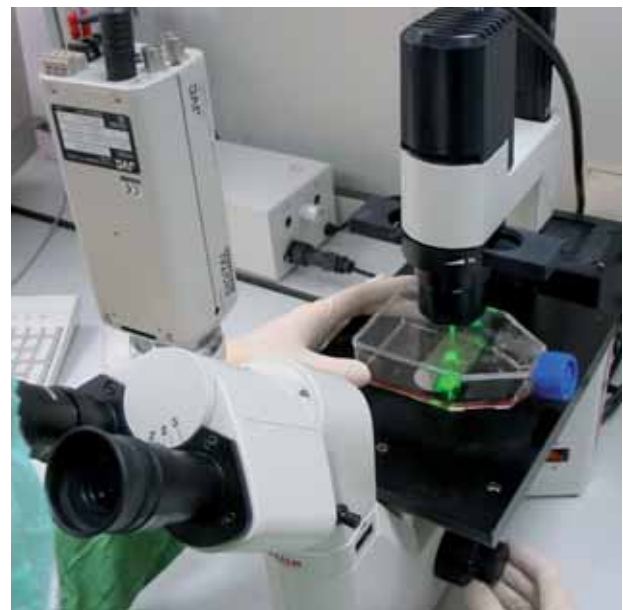
Image of a different case.



Image MRI of lumbar spine before treatment.



Different case MRI after treatment.



Laboratory samples.

Infiltration Technique:

Infiltration, including from the opposite side, if necessary;

Interval of between one week and 10 days between each session

12-14 sessions in total

4-5 cm from the line of the spinous apophysis

- Needle in latero-medial orientation

- Gradual release of the gaseous mixture (due to pain from expansion of the gasses).

The oxygen-ozone mixture was introduced into the paravertebral region at the height affected by the disc disease. The parts of the disc to be treated were identified through the normal anatomical methods, in some cases ultrasound was used in this process (Brina). Having identified the area of the disc to be treated, the area is thoroughly disinfected and the infiltration with the oxygen-ozone mixture performed under aseptic conditions, using the methods and dosages described above.



Insertion of stem cells.

These infiltrations were performed in the Donetsk Centre under the direction of Prof. A.G. Popandopulo.

After the last session of ozone treatment, bone marrow was harvested from the patient's iliac crest (photos 2-3-4).

80-100 ml of bone marrow was harvested under aseptic conditions from the iliac crest, with the addition of 625 units/ml of heparin (Darnytsa, Ukraine). The sample material was brought to the gradient HISTOPAQUE-1077, density 1.077 g/ml (Sigma, USA) and centrifuged for 30 minutes at 1500 rpm. The mononuclear cells obtained were then collected with a pipette and washed 3-4 times in Hanks' solution (Biolot, Russia) using centrifugation at 1000 rpm. for 14 minutes. In this way, a suspension of mononuclear bone marrow cells was obtained, and this was sown in culture dishes, each with a surface area of 75 cm² (Corning-Costar, USA) at a concentration of 2-5x10⁶ cells per dish.

Patient Scheme

Age	Diagnosis
54	Chronic disk syndrome L4-5: L5-S1 on the right
41	Chronic diskogenic lumbar ischialgia on the right
48	Chronic disk syndrome L5-S1 on the right
49	Chronic disk syndrome L5-S1 on the left
34	Chronic diskogenic myeloradiculopathy L5-S1 on the left
63	Chronic dorsal. Vertebrogenous double lumboischialgia
34	Chronic disk syndrome L5-S1 on the right

The MSK cells were cultured in a DMEM/F12 mixture, 1:1 (Sigma, USA) with the addition of 10% embryonic bovine serum (Biolot, Russia), 0.75 mg/ml glutamine (Institute of Poliomyelitis and Viral Encephalopathy, Russia), 2 ng/ml of fibroblast based growth factor (Sigma, USA) and 100 U/ml penicillin and streptomycin (Darnytsa, Ukraine), in an incubator (Jouan, France) at 37° C, 5% CO₂ and 95% atmospheric humidity. The culture medium was renewed every 3-4 days.

As a result of this process, a multiplication of MSK cells was obtained in the culture. A monolayer was obtained containing the following typical cells: CD105 + / CD73 + / CD90 (> 95%) and CD45-/CD34-/CD14- (<2% positive)

Thirty days after the oxygen-ozone infiltrations, an infiltration of 10 million autologous stem cells (MSK) was carried out on the same part of the same disc.

Results

Patients with chronic pain have benefited of the disappearance of the Lasègue. The only patient with paralysis of the EPA presented a significant improvement in dorsiflexion of the big toe.

At the end of treatment we could identify two groups of patients:

- Patients with chronic pain who have presented a complete remission of symptomatology after two weeks.

- Patients with acute pain who have had an immediate improvement, but who have had mild recurrence of symptomatology several days after the treatment, in contrast to the patients with chronic pain who, as mentioned above, tested completely negative on the Lasègue Test; and the only patient with paralysis of the EPA who showed considerable improvement in the strength of dorsiflexion of the big toe.

It should be noted that although implanting stem cells into the spinal canal, was a possibility, in this first experiment we preferred to investigate paravertebral infiltration.

The results are demonstrated by the following graphs.

Graph (a) shows improvement in sciatica symptomatology

Graph (b) shows the degree of muscular contraction and the resulting level of lower back pain.

All patients underwent spinal lumbar MRI scans, and as well as highlighting a reduction in the volume of hernia material, these also showed an improvement in the height of the intervertebral disc as well as an improvement in the trophism of the disc itself.

Conclusions

The paravertebral use of ozone in the treatment of disc disease is already well established, and the positive experiences of the orthopaedic surgeons and neurosurgeons who have been using the technique for years and achieving excellent results have been well documented in the literature.

The use of autologous stem cells in orthopaedic disorders is now a reality. However, the combination of the two techniques to increase the thickness and improve the quality of the disc has only received very limited exposure in the literature. In fact, there are no reported studies on the combined use of oxygen-ozone infiltrations and stem cell implantation in treating diseases affecting annulus fibrosus.

The investigations and studies carried out under the direction of Prof. Popandopulo of Donetsk have shown that when the two techniques are used in conjunction on the same patient, there is improved trophism of the diseased disc. MRI scans did not show a significant increase in the height

or thickness of the disc, though some MRI images showed that some patients' discs had changed, and in some cases showed improved trophism and were even a few millimetres thicker. Though our findings are not significant and there is no consistent follow-up work, the improvement in clinical symptoms, the reduction in the volume of hernia material after just a single combined infiltration with stem cells and ozone (not a course of treatment with weekly infiltrations as per protocol) make it clear that used together, an oxygen-ozone mix and stem cells act synergistically and rapidly on the chain of prostaglandins to eliminate back pain, and moreover produce a massive action on angiogenesis, and the restoration of protein binding in annulus (the latter was demonstrated by MRI images after treatment).

Finally, we believe that my idea needs to be carried forward and should eventually lead to infiltration with ozone-oxygen and stem cells implantation directly into the disc. This is the method this study group is carrying out.

Bibliography

- Jandial R, Aryan HE, Park J et al. Stem cell-mediated regeneration of the intervertebral disc: cellular and molecular challenges// *Neurosurg. Focus* – 2008- Vol. 24, ?3 – 4. – P. 1-5
- Haug J.I, Kazmi N, Dubhakula M.M et al. Chondrogenic potential of progenitor cells derived from human bone marrow and adipose tissue: a patient – matched comparison// *J Orthop Res.* – 2005. – Vol. 23. – P. 1383-1389
- Fan Yang, Victor YL Leung, Keith DK Luk et al. Mesenchymal Stem Cells Arrest Intervertebral Disc Degeneration Through Chondrocytic Differentiation and Stimulation of Endogenous Cells A Mouse Model Study// *Molecular Therapy* – 2009 – Vol 17 – P. 1959-1966
- Leung V.Y, Chan D, Cheung K.M. Regeneration of intervertebral disc by mesenchymal stem cells: potentials, limitations and future direction// *Eur Spine J.* – 2006. – Vol. 15, suppl. 3. – P. 406-413
- Paesold G, Nerlich A.G, Boos N. Biological treatment strategies for disc degeneration: potentials and shortcomings// *Eur Spine J.* – 2007. – Vol. 16. – P. 447-468
- J. Ciacci, A. Ho, C.P. Ames et al. Stem cell horizons in intervertebral disc degeneration//*Stem Cells and Cloning: Advances and Applications* – 2008 – Vol. 1. – P. 31-39
- Le Visage C, Kim S, Tatento K et al Interaction of human mesenchymal stem cells with disc cells: changes in extracellular matrix biosynthesis// *Spine.* – 2006. – Vol. 32. – P. 2036-42
- Stephen M. Richardson and Judith A. Hoyland. Stem cell regeneration of degenerated intervertebral discs: Current status//*Current Pain and Headache Reports.* – 2008 – Vol. 12, ? 2 – P. 83-88
- Daisuke Sakai, Joji Mochida, Toru Iwashina. Regenerative effects of transplanting mesenchymal stem cells embedded in atelocollagen to the degenerated intervertebral disc// *Biomaterials.* – 2006 – Vol. 27, issue 3. – P. 335 – 345
- Frank L. Acosta Jr., M.D.; Jeffrey Lotz, Ph.D.; Christopher P. Ames, M.D. The potential role of mesenchymal stem cell therapy for intervertebral disc degeneration: a critical overview// *Neurosurg. Focus* – 2005- Vol. 19 – P. 1-6.
- L. Brina, P.C. Villani: Ultrasound imaging in the treatment of lumbar herniated disc by paraarterial ozone injection. *The Italian Journal of oxygen-ozonotherapy* 2: 147-152, 2003.

Luigi Brina, MD
Orthopaedics 2^ San Paolo Bari
Via Caposcardicchio, 6
Modugno, Bari, Italy
E-mail: info@luigibrina.it

Lumbar Disc Disease: 10 years of Experience in Paravertebral Infiltration with Oxygen-Ozone Mix

L. BRINA

Orthopaedics 2^ San Paolo Bari, Italy

Key words: lumbar disc disease, paravertebral infiltration, ozigen-ozone

The increase in lumbar disease (discopathy, herniated discs and resultant back pain, sciatica-cruralgia) in recent decades has led to an exponential increase in health spending due to absence from work, cost of medication, physiotherapy and increased hospital bed occupancy. Time in hospital is required to reduce the duration of acute symptomatology and perform diagnostic tests. With the remission of symptoms and the completion of the diagnostic phase, decisions need to be taken to decide the best way to treat the disease: surgically, with an orthopaedic brace, physiotherapy, medication or a combination of the above. Pharmacotherapy has shown the usefulness of the alpha lipoic acid molecule in reducing symptoms of root inflammation. However, surgery may be required urgently when neurological deficits have occurred in the last 24 hours. There is a clear need to reduce the length of hospital stays and find an alternative to expensive new drugs that are being used more and more frequently (Tramadol).

Against this panorama, the use of paravertebral ozone infiltrations, with its ability, even after just one infiltration, to produce an immediate reduction in pain, and negative or improved Lasègue and Wasseramann tests, which reveal the degree of root compression becomes an interesting option. Ozone also relaxes the muscles enough to allow a rapid return to a standing position, permitting an earlier return to work for the patient.

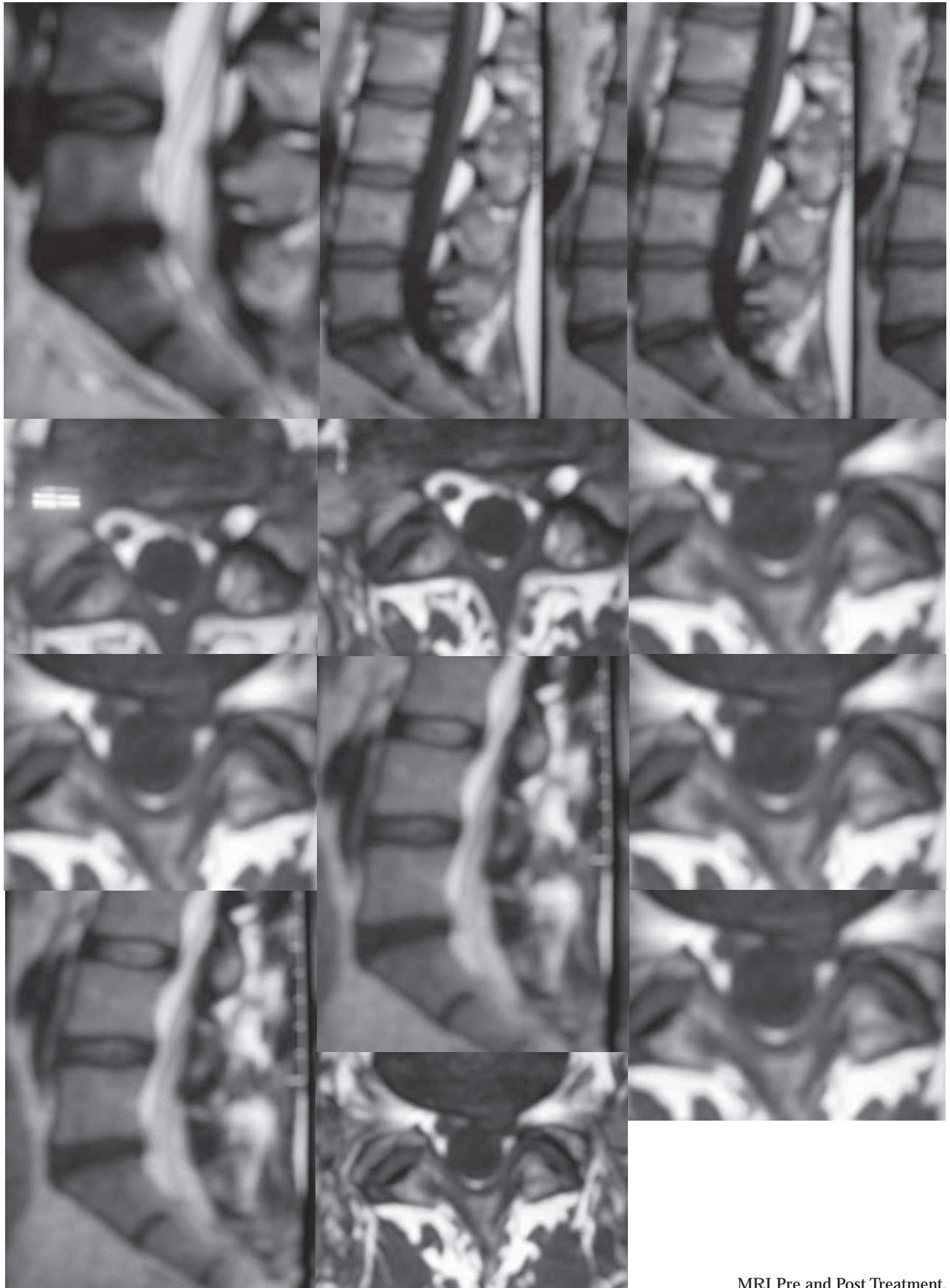
Paravertebral infiltration with oxygen-ozone mixture requires a thorough prior investigation using MRI scans and radiography (x-rays of the lumbar spine in the upright position and in dynamic projections in maximum flexion and extension of the spine). Careful clinical evaluation of the patient is required to identify the height at which the spine requires treatment. Above all, a precise methodology must be followed for the microsurgical process, observing strict infiltration protocols.

It should be emphasised that CT and fat saturation MRI scans show post-surgical fibrosis. The fibrosis is responsible for all the symptoms of back pain associated with post-discectomy instability, so there is a clear need to find a less invasive, more studied technique, that will not limit the mechanics of the vertebra-disc segment. The benefits of treating disc herniation or degenerative disc disease with paravertebral infiltration of oxygen-ozone mixture have already been highlighted by other authors (Alexander). Using surgery and other methods, over time, recurrent disc herniation can occur, as well as adhesion phenomena, post-surgical scarring and fibrosis, that then require, as already mentioned, new surgical interventions which can in turn produce acute symptomatology of spinal instability.

Ozone acts as:

- neoangiogenesis of the peridural vessels
 - anti-inflammatory (breaking the chain of prostaglandins that cause pain)
 - relaxant action of ozone on intervertebral disc
- Neof ormation of proteins and fibrocytes in the annulus.

Recently, Popandopulo and Brina have shown that using a combination of ozone and stem cells increases the tropism of the disc. It is essential at this point to define the anatomy of the intervertebral lumbar disc and its relationship with the vertebra itself (cortical spongy-bone and cartilaginous plates); disc: fibrocartilaginous ring (fibrous-elastic in the peripheral region); cartilage on the inside; the anulus consists of concentric lamellae of fibrous tissue. Vascularisation of the disc, also of extreme importance, is provided by blood vessels that enter and exit the disc through perforations in the central area of the cartilaginous plate. These blood vessels gradually atrophy over the first three decades of life, and subsequently vascularisation is



MRI Pre and Post Treatment.

provided by the lymphatic canals and the circulation of extracellular fluid.

The anatomy of the diseased disc is characterized by:

- reduced definition of the boundary between the outer part of the nucleo pulposus and the inner annulus.
- progressive loss of elasticity of the annulus fibrosus
- concentric circumferential injury beginning from the periphery of the annulus
- increase in size, extension and progression (like the domino effect) of damage radiating both towards the periphery and in towards centre of the annulus (corresponding to the posterior region of the disc)
- atrophy of the blood vessels of the disc's central plates

Techniques:

- Intradiscal
- Paravertebral
- Small and large autohaemotransfusion

The technique of paravertebral infiltration of the disc that we use is as follows (including ultrasound guidance).

- 2 doses of 10 ml, in the side to be treated with a syringe and 27G needle
- Dosage: 12-14 micrograms / ml ozone

Infiltration technique:

- infiltration from the opposite side if necessary
- interval of one week to 10 days between the individual sessions
- 12-14 sessions in total.

Infiltration technique:

- 4-5 cm from the line of the spinous apophysis
- Needle in latero-medial orientation
- Gradual release of the gaseous mixture (due to pain from expansion of the gasses).

Materials and methods

From 2001 to 2009, we treated about 500 patients at our centre, suffering from diseases of the spine including sciatica, cruralgia, back pain or with symptoms of symptoms of canal stenosis. In this last case, patients were informed of the possibility that the symptoms may not subside, or that any reduction in pain may only be slight. In other cases, however, patients recovered to the point of being able to walk unaided, and in two cases there was complete regression of paresis of the PRS.

Most of our patients were between 35 and

60, but we also treated older patients with serious degenerative diseases of the spine. Our best results have been achieved with patients who have come to us in the acute phase, with a positive Lasègue Test at 30 degrees.

These patients showed immediate remission of symptomatology after paravertebral infiltration. With all these patients we implemented a pharmaceutical wash-out, whereby they were discouraged from using pain killers during the treatment period.

All patients underwent lumbar spinal MRI scans after treatment. It was important for us to check carefully patients' medical records for any signs of cardiovascular or circulatory diseases that could advise against treatment. Each patient was given an ECG before starting the paravertebral ozone injections.

Routine blood and coagulation tests are also important in my opinion to avoid haematoma formation in the area where the infiltration is to be carried out. Patients are required to sign a clear, careful, precise and informative consent form before receiving treatment.

Case studies of two young patients

Patient 1, 35 years old with the big herniated L5-S1

MRI scans before and after treatment show disappearance of the hernia.

Patient 2 Herniated L4-L5 coronal MRI images. Reduction of the hernia in this case too.

Conclusions

The use of paravertebral ozone infiltrations in the treatment of disc disease is already well known, as are the peculiarities of the results. Orthopaedic surgeons and neurosurgeons have used the technique for years with excellent results. Recently, the method has been combined with the use of stem cells.

The two techniques have been used in conjunction with the aim of increasing the thickness and quality of the disc, but there is still only limited coverage of this research in the literature. Studies of oxygen-ozone infiltrations combined with stem cells in treating disease of the annulus fibrosus are not reported in the literature.

Studies and experimentation conducted by Prof. Popandopulo in Donetsk, Ukraine, have shown that the use of the two techniques together in a patient improves trophism of the diseased disc. We have no MRI evidence of a significant increase in the height of the disc, although some MRI images do show that the discs of our patients

have changed, becoming more trophic, and even a few millimeters higher. A mixture of oxygen and ozone infiltrated paravertebrally has been shown to reverse completely the symptomatology, and reduce the size of the hernia material.

Luigi Brina, MD
Orthopaedics 2^ San Paolo Bari
Via Caposcardicchio, 6
Modugno, Bari, Italy
E-mail: info@luigibrina.it

May Oxygen-Ozone Therapy Improves Cardiovascular Disorders?

V. Bocci^{1,*}, V. Travaglini² and I. Zanardi²

¹Department of Physiology, ²Department of Pharmaceutical Chemistry and Technology, University of Siena, Italy

Abstract: In an aging population vascular disorders well exemplified by the chronic limb ischemia, chronic heart failure, cerebral ischemia and age-related macular degeneration represent a serious medical and socio-economical problem. While there is always a not easily identifiable first pathogenic noxa, all of these diseases are characterized by ischemia, chronic inflammation and tissue degeneration. Orthodox medicine has provided several optimal drugs targeting various pathological situations but, even with their concomitant applications, it is not possible to reduce the chronic oxidative stress. Here it is proposed to associate the approach of ozonated autohemotherapy as a modifier of the biological response capable to block the pathological progress.

Key Words: Chronic limb ischemia, chronic heart failure, ictus, age-related macular degeneration, chronic oxidative stress, ozone therapy.

INTRODUCTION

Cardiovascular diseases including chronic limb ischemia (CLI), chronic heart failure (CHF) and cerebrovascular ischemia (CVI) not only compromise a productive life but markedly increase health-care costs and eventually represent the first cause of death. In affluent Countries, an excessive and incorrect diet, sedentary lifestyle, stressful working conditions and genetic factors are the main causes responsible for enhancing atherosclerosis, obesity and diabetes, premonitory of vascular progressive disorders. The complexity of the metabolic disorders has been aptly summarized by the concept of the "metabolic syndrome" [1,2]. The atrophic form of age-related macular degeneration (A-RMD) with the initial ischemia of the choroidal vessels, the progressive degeneration of the retinal pigment epithelium (RPE) and the retinal photoreceptors death can also be included, because increasing the oxygenation of the retina, which has a very high oxygen consumption, has a decisive relevance [3].

Orthodox medicine has provided a series of antidiabetics, antihypertensive agents, antiplatelet and antithrombotic drugs, statins possibly associated with etizimide or/and fibrates. All of these drugs, by normalizing the glycemia, arterial pressure, hypercoagulability, cholesterol and low-density lipoproteins levels are able to delay ischemic episodes, particularly when these drugs are associated to a moderate diet and physical activity.

Although the life-span of cardiovascular patients has been markedly prolonged, the pathological process remains active because of the ensuing chronic inflammation at vascular level, which provokes a chronic oxidative stress due to an imbalance between either an excess of oxidant molecules or/and the progressive weakening of the natural antioxidant

capacity [4]. Among the formers there are the reactive oxygen species (ROS) represented by anion superoxide (O_2^-), hydrogen peroxide (H_2O_2), possibly hydroxyl radicals (OH), peroxynitrite (ONOO⁻), and hypochlorous acid (HClO). Moreover, the peroxidation process, the complement activation, the release of coagulation, metalloproteinases and growth factors, thromboxane A and leukotrienes, cytokines such as tumor necrosis factor α (TNF α), interferon γ and interleukins (IL-1, IL-2, IL-6) perpetuate the inflammatory process.

The progressive release of toxic compounds and of abnormal concentrations of pro-inflammatory cytokines is responsible for cell death or degeneration or a proliferative dysregulation leading to a steady unbalance of the redox state owing to the cell inability to efficiently neutralize the excess of oxidants and toxic compounds. Thus the chronic inflammation worsens with time, possibly becoming irreversible unless we can dampen the process and restore a normal redox state. A vicious imbalance between oxidants and antioxidants is now firmly established in atherosclerosis, diabetes, ischemia, nephropathies, hyperhomocysteinemia, neurodegeneration, infections, autoimmune diseases, asthma, chronic obstructive pulmonary disease and cancer, just to name the most common pathologies. Unless we can interrupt this imbalance a more or less rapid progression is unavoidable. Numerous strategies potentially capable to reduce or contain the oxidative stress in these diseases have been devised as follows:

THE STATE OF THE ART

- 1). Administration of corticosteroids is one of the most frequently used procedures for reducing inflammation. Corticosteroids have been and are being used in a wide variety of pathologies ranging from dermatitis to brain tumors. Unfortunately the anti-inflammatory effect is temporary and serious adverse effects (diabetes, osteonecrosis, osteoporosis, Cushing's syndrome, glaucoma) can ensue with broad immunosuppression

*Address correspondence to this author at the Emeritus Professor of Physiology at the University of Siena, Italy Department of Physiology, University of Siena, Via Aldo Moro 2, 53100 Siena, Italy; Tel: +39 (0)577 234226; Fax: +39 (0) 577 234219; Email: bocci@unisi.it

- [5]. Moreover glucocorticoid resistance may result from a sustained reduction of histone deacetylase-2.
- 2). Non-steroidal anti-inflammatory drugs (NSAID) are also widely used mostly to overcome prostaglandin-related pain states. Different types of drugs can block either or both COX-1 and COX-2 isoforms and, they are effective analgesics, but, a prolonged use may cause gastric ulcerations and undesirable cardiovascular events [6,7].
 - 3). Administration of allopurinol improves endothelial dysfunction in CHF [8]. A randomized, double-blind crossover study on 11 patients with NYHA class II-III chronic heart failure received 300 mg allopurinol daily versus placebo. Allopurinol appeared to have inhibited xanthine oxidase thus reducing oxidative stress, a finding also supported by the significant reduction of plasma malondialdehyde.
 - 4). Inhibition of NADPH oxidase present in the plasma membrane of phagocytes [9], by limiting the production of superoxide, may reduce oxidative stress, but a direct action remains a difficult pharmacological problem with the further risk of increased bacterial infections.
 - 5). There is good evidence that inhibition of the renin-angiotensin system can reduce cardiovascular events [10]. Angiotensin-converting enzyme (ACE) inhibitors and Ang-II receptor antagonists associated with diuretics are broadly used drugs for effectively reducing blood pressure and partly inhibiting NADPH oxidase. Interestingly, several other antihypertensive drugs do not improve the antioxidant status in patients [11].
 - 6). The inhibition of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (or statins), the key enzyme of cholesterol biosynthesis may be beneficial [12]. Surprisingly statins, not only are able to lower serum cholesterol levels and increase the number of hepatic LDL receptors, but they are able to modulate pathophysiologic processes in patients with acute coronary syndromes [13]. By blocking the synthesis of critical isoprenoid intermediates [14], they express other effects such as a limited inhibition of NADPH oxidase, the increased expression of endothelial NO synthase and of tissue-type plasminogen activator, while they inhibit the expression of plasminogen activator inhibitor and endothelin-1. Indeed this "miracle drug" [15] seems to reduce inflammation, inhibits lymphomas progression [16] and the inflammatory components of multiple sclerosis [17]. Although rarely, statins may provoke rhabdomyolysis [18].
 - 7). It has been claimed that an excessive production of superoxide could be inhibited by long-term administration of L-arginine, which is the substrate for NO synthesis [19,20]. This approach and autohemotherapy may also reduce the many complications observed in sickle-cell anemia patients [21].
 - 8). An excessive production of superoxide could be also quenched by the administration of superoxide-dismutase (SOD) or, better, of enzyme mimetics able to enter into the cells [22,23]. This is an interesting possibility because exogenous enzymes are antigenic and unable to enter into the cell. For some of these mimetics, the pharmacology and toxicity remain to be defined. Nonetheless, in order to investigate the therapeutic potential of these drugs, extensive clinical trials are warranted.
 - 9). The potential of inducing a therapeutic advantage through gene transfer of antioxidant enzymes has been taken into consideration in rabbits [24] but some relevant issues have to be solved prior to clinical application, including the understanding of the most suitable vectors, the feasibility and stability of gene delivery to different tissues or/and organs affected by the oxidant stress. Studies on longevity have shown some correlation with increased resistance to oxidative stress and an intriguing relationship among intracellular oxidants, *p66shc*, forkhead protein and *p53*. This aspect may become important but again we are not yet able to practically manipulate the *p66shc* gene [25,26].
 - 10). Whenever the case, the increase of homocysteine levels in the plasma must be inhibited because the auto-oxidation of its sulfhydryl group generates superoxide and hydrogen peroxide that are cytotoxic for the endothelium. Hyperhomocysteinemia can be kept under control by the daily administration of folic acid and vitamins B6 and B12 [27] that may allow a normalization of adenosine plasma levels [28].
 - 11). It is well known that an abnormal platelet aggregation has dire consequences and can be inhibited with a variety of antithrombotic agents from the old aspirin to the recent clopidogrel [29].
 - 12). Moreover the synthesis of pro-inflammatory auto-oxids and platelet aggregability can be inhibited by the administration (2-3 g daily) of n-3 PUFAs present in fish oil. These particular unsaturated fatty acids enhance the generation of 3-series prostaglandins and 5-series leukotrienes, which are anti-inflammatory [30,31].
 - 13). It is well known that oxidative stress due to formation of OH[•] is enhanced by free transition metals (Fe²⁺, Cu⁺). For some time chelation therapy with EDTA, deferoxamine and defasirox have been popular, but there is now a broad consensus to abandon this approach [32,33].
 - 14). A hybrid of K_{ATP} channel opener and nicotinamide nitrate (Nicorandil) was found to improve cardiac function in acute myocardial infarction by probably reducing ROS formation [34].
 - 15). The famous glycation reaction due to hyperglycemia leads to the formation of advanced glycation end

products (AGEs). The AGEs which are irreversible toxic compounds, deposited in the arterial wall can induce oxidative stress leading to diffused damages and accelerating the progression of diabetes type II, atherosclerosis, renal and retinal damage. Thus hyperglycemia and obesity must be avoided by regulating caloric intake, possibly adopting the norm of caloric restriction [35] with adequate nutrition and by adopting a correct life style without smoking or drinking alcohol and finding time for a daily physical exercise.

- 16). An excessive paracrine secretion of proinflammatory cytokines such as TNF- α , IL-1, IL-6, IL-8 at the sites of chronic inflammation has been demonstrated in rheumatoid arthritis, Crohn's disease, multiple sclerosis as well as in solid cancer, chronic viral diseases and in chronic pulmonary obstructive disease (COPD) to name a few pathologies. In about 2/3 of rheumatoid arthritis patients, a variety of TNF- α antagonists has significantly improved the clinical outcome by reducing inflammation [36], while antibodies to the other cytokines are far less efficacious. However, TNF- α inhibitors are hardly effective in other diseases with the further caveat that TNF- α antagonists cannot definitively block the disease progression and, with prolonged use, may increase the susceptibility to opportunistic infections [37]. It was postulated that CHF may be due to a continuous release of proinflammatory cytokines, but several clinical trials, aimed to neutralize TNF α in patients with fairly advanced CHF, have resulted in worsening the outcome. It was concluded that neutralization of TNF α alone was inadequate because IL-1 β and IL-6 may contribute to the inflammation [38-42]. It was then hypothesized that a new immunomodulation therapy based on treating patients with their own heavily oxidized blood may stop the progression of heart failure [43-46], but a more recent and ample evaluation, further discussed in detail, has yielded disappointing results [47]. At least in theory, immune suppression of the autoimmune reactions may be achieved by the administration of IL-10 and TGF- β 1 but to date this remains a possibility to be tested. CD25+ regulatory T cells (Tregs) may be able to control excessive immune responses but, as yet, potentially valid approaches are still in an experimental phase.
- 17). The discovery of "the cholinergic anti-inflammatory pathway" [48] has clarified that the nervous system can modulate immune responses in real time. The efferent vagus nerve can control the inflammatory response by inhibiting the α 7 subunit of the nicotinic acetylcholine receptors expressed on macrophages. Practical therapeutic modalities are being explored [49] for their possible application in the treatment of endotoxemia, myocardial depression and cancer.
- 18). For a long time, Dr. Richard Spears has pioneered the direct intravascular application of a high oxygen tension in ischemic tissues especially after coronary occlusion. He has proposed to use an extremely high concentration of oxygen dissolved in a saline solution as a more effective alternative to the hyperbaric oxygen chamber [50]. Anoxic myocardium needs oxygen, but the result depends upon a precise time interval as otherwise oxygen may aggravate the damage. Indeed, so far the reperfusion appeared safe, but a modest effect was observed only when reperfusion was performed < 6h from symptom onset. Similar to what may happen during a treatment with the hyperbaric oxygen chamber, an excessive oxygen concentration may well represent a double-edged sword [51].
- 19). The use of small interfering RNA (siRNA) is promising but, owing to the fact that the chronic oxidative stress is due to a multitude of factors, it will be necessary to first evaluate the most important target(s) and then assess the difficult problem of a suitable delivery system of the selected dsRNAs [52].
- 20). As the chronic oxidative stress is due to an imbalance between an excess of ROS and LOPs and a deficit of antioxidants, it has become obvious that a supplementary administration of antioxidants may eliminate the stress [4]. This idea has become a fashionable theme, amply discussed by vitaminologists with the risk of intoxicating patients with megadoses of Vitamin A, C and E plus selenium and zinc. A fair conclusion is that the recommended dietary allowance (RDA) of micronutrients, supplemented by a rich dietary intake of fresh fruit and vegetables, while it is important during growth or denutrition or cachexia, it may not have a crucial relevance in oxidative stress-related conditions. Indeed the evidence that it can be a real remedy remains controversial [53-67]. On the other hand, megadoses may be either toxic or counterproductive in the sense that, at the very least, they may inhibit the synthesis of heme-oxygenase-I (HO-I) as described by Peng *et al.* [68]. The following factors can explain why an excessive oral supplementation can be of scarce value:
 - i. the uncertainty of intestinal absorption. Usually the higher the vitamin dose, the lower is the percentage of absorption.
 - ii. the difficulty of maintaining a steady plasma level,
 - iii. the individual variability of metabolism and excretion,
 - iv. of utmost importance is the variable and limited uptake of antioxidants tightly regulated by either the cell membrane active- or facilitative-transport. The fashionable idea that intravenous injection of megadoses of vitamin C and reduced glutathione do miracles ought to be firstly well documented by pharmacological and clinical data. It is well known that cellular uptake of GSH is inefficient and that it is preferable to administer orally three times daily

600 mg of N-acetylcysteine (NAC) [69]. Moreover the lysine salt of NAC, N-acetylcysteyn (NAL), by forming a neutral pH when in solution, is more effective than NAC [70].

- v. the possibility that some antioxidant vitamins may exert oxidant activity.
- vi. the inability of vitamins to stimulate the synthesis of cellular antioxidant enzymes which represent the crucial defence. Thus the approach of an antioxidant supplementation must be well weighed and, although seems useful to administer an equilibrated amount, it remains doubtful if it can correct the chronic oxidative stress, unless the patient's organism has become unable to efficiently recycle dehydroascorbate and GSH disulphide to ascorbic acid and GSH. The problem with the chronic oxidative stress is not necessarily the lack of vitamins as much as the constant and elevated intracellular levels of oxidants accompanied by a chronic deficiency of reduced glutathione and thioredoxin due to the inability of producing sufficient reductive power. As beautifully exposed by Mendiratta *et al.* [71], Dickinson and Forman [72], and Wilson [73], low molecular weight thiol compounds and ascorbic acid play an essential role in many reactions "due to the ease with which they are oxidized, and the rapidity with which they can be regenerated." During a chronic inflammation, besides a reduced generation of ATP, the main problem is linked to the defective supply of the major electron donor, NADPH and of antioxidant enzymes such as GSH peroxidases (GSH-px), GSH reductases (GSH-rd) and GSH S-transferases (GST), the latter performing conjugation reactions able to neutralize toxic aldehydes. Obviously a concomitant deficit of SOD and of glucose 6-phosphate dehydrogenase (G6PD) is important while we have rarely observed a deficit of catalase. The crucial advance will come if it will be possible to reverse the chronic oxidative stress by actively inducing the upregulation of intracellular antioxidant enzymes

THE POSSIBILITY OF CORRECTING THE CHRONIC OXIDATIVE STRESS

This problem has been investigated during the last two decades and it has been clearly shown that a small oxidative stress due to hyperoxia as well as ROS [74-78] and, more recently, therapeutic doses of ozone [79-85] can upregulate the synthesis of antioxidant enzymes and of HO-1, which is now one of the most interesting enzymes because it has antioxidant and multifunctional protective activity [86-89]. It has become clear that any change of the external environment disturbs cell homeostasis, but if the stress (hyperthermia, hyperoxia, radiation, ischemia, xenobiotics and so on) is tolerable, or graduated in intensity, the cell can adapt to it and survive. On the other hand, if the stress exceeds the cell capabilities, the cell programmes its own death. This behaviour

is universally present from bacteria to fungi to plants to mammals and the concept of ischemic and oxidative preconditioning has been extensively discussed elsewhere [90]. Goldman [91] and Calabrese [92-2005] have presented examples of stimulatory responses following stimuli below the toxicological threshold and have coined the term "hormesis." As we have used small, precisely calculated and repeated ozone stresses [79,81,83], we have selected to use the term of *induced adaptation to a calibrated oxidative stress*.

There are several modalities but the most precise, where ozone and blood antioxidants can really stoichiometrically react is ozone therapy, under the form of a major autohemotherapy. The procedure consists in 2-3 weekly intravenous reinfusion of 200-250 ml of the patient's blood mixed with an equal volume of a gas mixture composed of medical-grade oxygen (~96%) and ozone (equivalent to progressively increasing ozone doses just five minutes after gently mixing the gas mixture with blood). We have adopted the strategy of slowly increasing the ozone dose from 4-5 mg up to 16-20 mg (200-250 ml of blood, respectively) to gradually improve the adaptation to the ozone stress. Thus, ozone induces a calibrated acute oxidative stress during which a number of well defined messengers (H₂O₂, 4-hydroxynonenal) interact with blood and parenchymal cells and induce the upregulation of antioxidant enzymes and HO-1.

The practice of autohemotherapy is some 40 years old and it has been performed in many Countries showing to be effective, atoxic and well-tolerated [93,94], especially in ARMD [3] and chronic limb ischemia [95] which are the most frequently treated disorders.

On the basis of the mechanism of action, ozone therapy can induce the following biological responses: a) it improves blood circulation and oxygen delivery to ischemic tissue owing to the concerted effect of NO and CO and an increase of intraerythrocytic 2,3-DPG level; b) by improving oxygen delivery, it enhances the general metabolism; c) it upregulates the cellular antioxidant enzymes and induces HO-1 and HSP-70; d) it induces a mild activation of the immune system and enhances the release of growth factors; e) it does not procure acute or late side effects; and, finally, it procures a surprising wellness in most of the patients, probably by stimulating the neuro-endocrine system. It does seem that ozone therapy acts as a biological response modifier on many targets gone astray because of a chronic inflammation. All of these biological modifications have been extensively discussed elsewhere [82,90,95] but it remains still uncertain whether some messengers present in the ozonated blood are able to stimulate the release of staminal cells in the patient's bone marrow. The mobilization of these cells would represent a crucial advantage in both CLI and CHF.

Nonetheless, this simple and inexpensive procedure has already yielded therapeutic results in CLI (grade 2-4 of Fontaine-Leriche's classification) superior to those achieved by orthodox medicine using the gold standard infusion of various prostanoids [96-106]. It appears likely that the exceptional versatility of the procedure makes it amenable to be

evaluated in CHF and ictus. In such a case, by simultaneously using a fibrinolytic agent (tissue plasminogen activator) with mild ozone therapy appears important to rapidly reduce hypoxia of the penumbral zone, thus minimizing further complications [107].

Why our very mild ozonetherapeutic modality should be effective when the Celacade™ system, thought to be an immune-modulation procedure based on the intragluteal injection of 10 ml of the CHF's patient blood (grade II and III of the New York Heart Association, functional classification) after its treatment with as much as 75 mg of ozone, plus UVA irradiation and heat stress at 42.5 °C has failed to improve the prognosis? This extremely harsh procedure proposed in 1997 [43] and supported by commercial interest was wrongly believed to reverse *in vivo* the prevalence of the T-helper type 1 (Th1)/T-helper type 2 (Th2) equilibrium and inhibit the vascular flogosis. Already in 1996 the "SIM-PADICO" trial programmed for improving the chronic limb ischemia was aborted [108] owing to the lack of a clinical result. However, it has been the final "ACCLAIM" trial published in 2008 [47] that has shown no real clinical improvement in 1213 CHF's patients treated for about 6 months with some 25 i.m. injections of their heavily oxidized blood. Although four critical comments [109-112] on the rationale of this approach have explained this failure, the improper use of an ozone dose at least 92-fold higher than the real therapeutic dose (from 20 µg/ml or 0.42 µmol/ml up to 80 µg/ml or 1.68 µmol/ml gas per ml of blood) as we have determined over the years [82-84] has compromised the future of ozone therapy. The misinterpretation of the real mechanisms of action such as vasodilatation, increased delivery of oxygen in ischemic tissue and upregulation of antioxidant enzymes and HO-1 has completely subverted the proposal of the physiological use of ozone as a valid medical drug.

In spite of this serious pitfall, it is believed that the ozonetherapeutic approach simultaneously applied with the broad range of orthodox drugs, may represent a promising approach for correcting the chronic oxidative stress and improving the uncertain prognosis of many patients. Clinical protocols had been prepared for the four mentioned vascular disorders and will be evaluated during the next two years. Any interested clinicians in this endeavour would receive our best assistance.

CONCLUDING REMARKS AND A PERSPECTIVE

There is a general consensus that vascular disorders such as CLI, CHF, ictus, and A-RMD are progressive diseases where the common denominators are the ischemia and an irreversible chronic inflammation. Owing to a number of concomitant negative factors, it is unlikely that the separate use of the available therapeutic strategies for reducing the chronic oxidative stress may succeed in improving the prognosis. The problem is complex because among obesity, diabetes, hypertension, and the likes there are substantial pathologic differences so that the clinician has the difficult task to select the best medicinal combination for achieving a benefit. Moreover, several of current drugs are too narrowly focused

and not all patients are able to be compliant. The basic aim is to decrease oxidative stress and it is felt that a judicious and simultaneous application of ozone therapy able to radically enliven the biological response is an approach that must be explored for the sake of many patients.

Just to mention a few modern approaches such as gene therapy, autologous transplantation of bone-marrow cells and administration of angiogenic factors, how ozonotherapy compares today with them? Ozone acts as a pro-drug: it dissolves almost instantly in the plasma, switches on a number of biochemical reactions and disappears after generating a number of messengers that, by reacting with a great variety of cells, allow the revival of important biological activities went astray during the preceding chronic oxidative stress. Thus, ozone acts as a natural drug which has a minimal cost, no side effects and procures useful clinical benefits that can be maintained with a bimonthly administration. Indeed this review has intended to inform clinicians of the existence of this neglected procedure.

REFERENCES

- [1] Laaksonen, D.E.; Niskanen, L.; Lakka, H.M.; Lakka, T.A.; Uusitupa, M. Epidemiology and treatment of the metabolic syndrome. *Ann. Med.*, **2004**, *36*, 332-346.
- [2] Eckel, R.H.; Grundy, S.M.; Zimmet, P.Z. The metabolic syndrome. *Lancet*, **2005**, *365*, 1415-1428.
- [3] Bocci V. In *Ozone. A new medical drug*; Bocci; Springer: Dordrecht, **2005**; pp.132-144.
- [4] Halliwell, B. Antioxidants in human health and disease. *Annu. Rev. Nutr.*, **1996**, *16*, 33-50.
- [5] Rhen, T.; Cidlowski, J.A. Antiinflammatory action of glucocorticoids-new mechanisms for old drugs. *N. Engl. J. Med.*, **2005**, *353*, 1711-1723.
- [6] FitzGerald, G.A.; Patrono, C. The coxibs, selective inhibitors of cyclooxygenase-2. *N. Engl. J. Med.*, **2001**, *345*, 433-442.
- [7] Beardsley S. Avoiding another Vioxx. *Sci. Am.*, **2005**, *292*, 16,18.
- [8] Farquharson, C.A.; Butler, R.; Hill, A.; Belch, J.J.; Struthers, A.D. Allopurinol improves endothelial dysfunction in chronic heart failure. *Circulation*, **2002**, *106*, 221-226.
- [9] Lambeth, J.D. NOX enzymes and the biology of reactive oxygen. *Nat. Rev. Immunol.*, **2004**, *4*, 181-189.
- [10] Schiffrin, E.L. Vascular and cardiac benefits of angiotensin receptor blockers. *Am. J. Med.*, **2002**, *113*, 409-418.
- [11] Baykal, Y.; Yilmaz, M.I.; Celik, T.; Gok, F.; Rehber, H.; Akay, C.; Kocar, I.H. Effects of antihypertensive agents, alpha receptor blockers, beta blockers, angiotensin-converting enzyme inhibitors, angiotensin receptor blockers and calcium channel blockers, on oxidative stress. *J. Hypertens.*, **2003**, *21*, 1207-1211.
- [12] Endo A. The discovery and development of HMG-CoA reductase inhibitors. *J. Lipid Res.*, **1992**, *33*, 1569-1582.
- [13] Spencer, F.A.; Allegrone, J.; Goldberg, R.J.; Gore, J.M.; Fox, K.A.; Granger, C.B.; Mehta, R.H.; Brieger, D. GRACE Investigators. Association of statin therapy with outcomes of acute coronary syndromes: the GRACE study. *Ann. Intern. Med.*, **2004**, *140*, 857-866.
- [14] Liao, J.K. Isoprenoids as mediators of the biological effects of statins. *J. Clin. Invest.*, **2002**, *110*, 285-288.
- [15] Roberts, W.C. The underused miracle drugs: the statin drugs are to atherosclerosis what penicillin was to infectious disease. *Am. J. Cardiol.*, **1996**, *78*, 377-378.
- [16] Katano, H.; Pesnicak, L.; Cohen, J.I. Simvastatin induces apoptosis of Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines and delays development of EBV lymphomas. *Proc. Natl. Acad. Sci. USA*, **2004**, *101*, 4960-4965.
- [17] Vollmer, T.; Key, L.; Durkalski, V.; Tyor, W.; Corboy, J.; Markovic-Plese S.; Preiningerova, J.; Rizzo, M.; Singh, I. Oral simvastatin treatment in relapsing-remitting multiple sclerosis. *Lancet*, **2004**, *363*, 1607-1608.

- [18] Lane, R.; Phillips, M. Rhabdomyolysis. *BMJ*, **2003**, *327*, 115-116.
- [19] Morris, C.R.; Kuypers, F.A.; Larkin, S.; Sweeters, N.; Simon, J.; Vichinsky, E.P.; Styles, L.A. Arginine therapy: a novel strategy to induce nitric oxide production in sickle cell disease. *Br. J. Haematol.*, **2000**, *111*, 498-500.
- [20] Gonzalez, F.M.; Shiva, S.; Vincent, P.S.; Ringwood, L.A.; Hsu, L.Y.; Hon, Y.Y.; Aletras, A.H.; Cannon, R.O. 3rd; Gladwin, M.T.; Arai, A.E. Nitrite anion provides potent cytoprotective and anti-apoptotic effects as adjunctive therapy to reperfusion for acute myocardial infarction. *Circulation*, **2008**, *117*, 2986-2994.
- [21] Bocci, V.; Aldinucci, C. Rational bases for using oxygenozonotherapy as a biological response modifier in sickle cell anemia and beta-thalassemia: a therapeutic perspective. *J. Biol. Regul. Homeost. Agents*, **2004**, *18*, 38-44.
- [22] Fontana, L.; McNeill, K.L.; Ritter, J.M.; Chowienzyk, P.J. Effects of vitamin C and of a cell permeable superoxide dismutase mimetic on acute lipoprotein induced endothelial dysfunction in rabbit aortic rings. *Br. J. Pharmacol.*, **1999**, *126*, 730-734.
- [23] Kirkham, P.; Rahman, I. Oxidative stress in asthma and COPD: antioxidants as a therapeutic strategy. *Pharmacol. Ther.*, **2006**, *111*, 476-494.
- [24] Laukkanen, M.O.; Kivelä, A.; Rissanen, T.; Rutanen, J.; Karkkainen, M.K.; Leppanen, O.; Bräsen, J.H.; Ylä-Herttua, S. Adenovirus-mediated extracellular superoxide dismutase gene therapy reduces neointima formation in balloon-denuded rabbit aorta. *Circulation*, **2002**, *106*, 1999-2003.
- [25] Nemoto, S.; Finkel, T. Redox regulation of forkhead proteins through a p66shc-dependent signaling pathway. *Science*, **2002**, *295*, 2450-2452.
- [26] Pandolfi, S.; Bonafè, M.; Di Tella, L.; Tiberi, L.; Salvioli, S.; Monti, D.; Sorbi, S.; Franceschi, C. p66(shc) is highly expressed in fibroblasts from centenarians. *Mech. Ageing Dev.*, **2005**, *126*, 839-844.
- [27] Das, U.N. Folic acid says NO to vascular diseases. *Nutrition*, **2003**, *19*, 686-692.
- [28] Riksen, N.P.; Rongen, G.A.; Blom, H.J.; Russel, F.G.; Boers, G.H.; Smits, P. Potential role for adenosine in the pathogenesis of the vascular complications of hyperhomocysteinemia. *Cardiovasc. Res.*, **2003**, *59*, 271-276.
- [29] Blann, A.D.; Landray, M.J.; Lip, G.Y. ABC of antithrombotic therapy: An overview of antithrombotic therapy. *BMJ*, **2002**, *325*, 762-765.
- [30] Belluzzi, A.; Brignola, C.; Campieri, M.; Pera, A.; Boschi, S.; Miglioli, M. Effect of an enteric-coated fish-oil preparation on relapses in Crohn's disease. *N. Engl. J. Med.*, **1996**, *334*, 1557-1560.
- [31] Mori, T.A.; Woodman, R.J.; Burke, V.; Puddey, I.B.; Croft, K.D.; Beilin, L.J. Effect of eicosapentaenoic acid and docosahexaenoic acid on oxidative stress and inflammatory markers in treated-hypertensive type 2 diabetic subjects. *Free Radic. Biol. Med.*, **2003**, *35*, 772-781.
- [32] Knudson, M.L.; Wyse, D.G.; Galbraith, P.D.; Brant, R.; Hildebrand, K.; Paterson, D.; Richardson, D.; Burkart, C.; Burgess, E. Program to Assess Alternative Treatment Strategies to Achieve Cardiac Health (PATCH) Investigators. Chelation therapy for ischemic heart disease: a randomized controlled trial. *JAMA*, **2002**, *287*, 481-486.
- [33] Atwood, K.C.; Woelckner, E.; Baratz, R.S.; Sampson, W.I. Why the NIH Trial to Assess Chelation Therapy (TACT) should be abandoned. *Medscape J. Med.*, **2008**, *10*, 115.
- [34] Ono, H.; Osanai, T.; Ishizaka, H.; Hanada, H.; Kamada, T.; Onodera, H.; Fujita, N.; Sasaki, S.; Matsunaga, T.; Okumura, K. Nicorandil improves cardiac function and clinical outcome in patients with acute myocardial infarction undergoing primary percutaneous coronary intervention: role of inhibitory effect on reactive oxygen species formation. *Am. Heart J.*, **2004**, *148*, 611-617.
- [35] Fontana, L.; Meyer, T.E.; Klein, S.; Holloszy, J.O. Long-term calorie restriction is highly effective in reducing the risk for atherosclerosis in humans. *Proc. Natl. Acad. Sci. USA*, **2004**, *101*, 6659-6663.
- [36] Maini, R.N.; Feldmann, M. How does infliximab work in rheumatoid arthritis? *Arthritis. Res.*, **2002**, *4*, S22-S28.
- [37] Emery, P. Treatment of rheumatoid arthritis. *BMJ*, **2006**, *332*, 152-155.
- [38] Bolton, A.E. Biologic effects and basic science of a novel immune-modulation therapy. *Am. J. Cardiol.*, **2005**, *95*, 24C-29C.
- [39] Gullestad, L.; Aukrust, P. Review of trials in chronic heart failure showing broad-spectrum anti-inflammatory approaches. *Am. J. Cardiol.*, **2005**, *95*, 17C-23C.
- [40] Mann, D.L. Targeted anticytokine therapy and the failing heart. *Am. J. Cardiol.*, **2005**, *95*, 9C-16C.
- [41] Torre-Amione, G. Immune activation in chronic heart failure. *Am. J. Cardiol.*, **2005**, *95*, 3C-8C.
- [42] El-Menyar, A.A. Cytokines and myocardial dysfunction: state of the art. *J. Card. Fail.*, **2008**, *14*, 61-74.
- [43] Bulmer, J.; Bolton, A.E.; Pockley, A.G. Effect of combined heat, ozonation and ultraviolet irradiation (VasoCare) on heat shock protein expression by peripheral blood leukocyte populations. *J. Biol. Regul. Homeost. Agents.*, **1997**, *11*, 104-110.
- [44] Babaei, S.; Stewart, D.J.; Picard, P.; Monge, J.C. Effects of VasoCare therapy on the initiation and progression of atherosclerosis. *Atherosclerosis*, **2002**, *162*, 45-53.
- [45] Torre-Amione, G.; Sestier, F.; Radovancevic, B.; Young, J. Effects of a novel immune modulation therapy in patients with advanced chronic heart failure: results of a randomized, controlled, phase II trial. *J. Am. Coll. Cardiol.*, **2004**, *44*, 1181-1186.
- [46] Torre-Amione, G.; Sestier, F.; Radovancevic, B.; Young, J. Broad modulation of tissue responses (immune activation) by celcade may favorably influence pathologic processes associated with heart failure progression. *Am. J. Cardiol.*, **2005**, *95*, 30C-37C.
- [47] Torre-Amione, G.; Anker, S.D.; Bourge, R.C.; Colucci, W.S.; Greenberg, B.H.; Hildebrandt, P.; Keren, A.; Motro, M.; Moyé, L.A.; Otterstad, J.E.; Pratt, C.M.; Ponikowski, P.; Rouleau, J.L.; Sestier, F.; Winkelmann, B.R.; Young, J.B. Advanced Chronic Heart Failure CLinical Assessment of Immune Modulation Therapy Investigators. Results of a non-specific immunomodulation therapy in chronic heart failure (ACCLAIM trial): a placebo-controlled randomised trial. *Lancet*, **2008**, *371*, 228-236.
- [48] Tracey, K.J. The inflammatory reflex. *Nature*, **2002**, *420*, 853-859.
- [49] Pavlov, V.A.; Tracey, K.J. The cholinergic anti-inflammatory pathway. *Brain Behav. Immun.*, **2005**, *19*, 493-499.
- [50] O'Neill, W.W.; Martin, J.L.; Dixon, S.R.; Bartorelli, A.L.; Trabattini, D.; Oemrawsingh, P.V.; Atsma, D.E.; Chang, M.; Marquardt, W.; Oh, J.K.; Krucoff, M.W.; Gibbons, R.J.; Spears, J.R. AMIHOT Investigators. Acute Myocardial Infarction with Hyperoxemic Therapy (AMIHOT): a prospective, randomized trial of intracoronary hyperoxemic reperfusion after percutaneous coronary intervention. *J. Am. Coll. Cardiol.*, **2007**, *50*, 397-405.
- [51] Bocci V. Comparative clinical studies between HOT and ozone-therapy in selected chronic pathologies are urgently needed. *Arch. Med. Res.*, **2006**, *37*, 919.
- [52] Castanotto, D.; Rossi, J.J. The promises and pitfalls of RNA-interference-based therapeutics. *Nature*, **2009**, *457*, 426-433.
- [53] Melton, L. The antioxidant myth. *New Scientist*, **2006**, *191*, 40-43.
- [54] Hennekens, C.H.; Buring, J.E.; Peto, R. Antioxidant vitamins-benefits not yet proved. *N. Engl. J. Med.*, **1994**, *330*, 1080-1081.
- [55] Packer, L.; Witt, E.H.; Tritschler, H.J. Alpha-lipoic acid as a biological antioxidant. *Free Radic. Biol. Med.*, **1995**, *19*, 227-250.
- [56] Omenn, G.S.; Goodman, G.E.; Thornquist, M.D.; Balmes, J.; Cullen, M.R.; Glass, A.; Keogh, J.P.; Meyskens, F.L.; Valanis, B.; Williams, J.H.; Barnhart, S.; Hammar, S. Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease. *N. Engl. J. Med.*, **1996**, *334*, 1150-1155.
- [57] Clinton, S.K. Lycopene: chemistry, biology, and implications for human health and disease. *Nutr. Rev.*, **1998**, *56*, 35-51.
- [58] McCall, M.R.; Frei, B. Can antioxidant vitamins materially reduce oxidative damage in humans? *Free Radic. Biol. Med.*, **1999**, *26*, 1034-1053.
- [59] Bender, D.A. Daily doses of multivitamin tablets. *BMJ*, **2002**, *325*, 173-174.
- [60] Vivekananthan, D.P.; Penn, M.S.; Sapp, S.K.; Hsu, A.; Topol, E.J. Use of antioxidant vitamins for the prevention of cardiovascular disease: meta-analysis of randomised trials. *Lancet*, **2003**, *361*, 2017-2023.
- [61] Seifried, H.E.; McDonald, S.S.; Anderson, D.E.; Greenwald, P.; Milner, J.A. The antioxidant conundrum in cancer. *Cancer Res.*, **2003**, *63*, 4295-4298.

A Comprehensive Approach for Vascular Disorders

Cardiovascular & Haematological Disorders-Drug Targets, 2009, Vol. 9, No. 2 7

- [62] Polidori, M.C.; Mecocci, P.; Levine, M.; Frei, B. Short-term and long-term vitamin C supplementation in humans dose-dependently increases the resistance of plasma to ex vivo lipid peroxidation. *Arch. Biochem. Biophys.*, **2004**, *423*, 109-115.
- [63] Ames, B.N. A role for supplements in optimizing health: the metabolic tune-up. *Arch. Biochem. Biophys.*, **2004**, *423*, 227-234.
- [64] Editorial. Lack of evidence for benefit of multivitamins. *Lancet*, **2006**, *367*, 1704.
- [65] Mudway, I.S.; Behndig, A.F.; Helleday, R.; Pourazar, J.; Frew, A.J.; Kelly, F.J.; Blomberg, A. Vitamin supplementation does not protect against symptoms in ozone-responsive subjects. *Free Radic. Biol. Med.*, **2006**, *40*, 1702-1712.
- [66] Harrison, F.E.; May, J.M. Vitamin C function in the brain: vital role of the ascorbate transporter SVCT2. *Free Radic. Biol. Med.*, **2009**, doi:10.1016/S0140-6736(06)68743-6, in press.
- [67] Roberts, L.J. 2nd; Oates, J.A.; Linton, M.F.; Fazio, S.; Meador, B.P.; Gross, M.D.; Shyr, Y.; Morrow, J.D. The relationship between dose of vitamin E and suppression of oxidative stress in humans. *Free Radic. Biol. Med.*, **2007**, *43*, 1388-1393.
- [68] Peng, J.; Jones, G.L.; Watson, K. Stress proteins as biomarkers of oxidative stress: effects of antioxidant supplements. *Free Radic. Biol. Med.*, **2000**, *28*, 1598-1606.
- [69] Bridgeman, M.M.; Marsden, M.; Selby, C.; Morrison, D.; MacNee, W. Effect of N-acetyl cysteine on the concentrations of thiols in plasma, bronchoalveolar lavage fluid, and lung tissue. *Thorax*, **1994**, *49*, 670-675.
- [70] Gillissen, A.; Jaworska, M.; Orth, M.; Coffiner, M.; Maes, P.; App, E.M.; Cantin, A.M.; Schultze-Werninghaus, G. Nacystelyn, a novel lysine salt of N-acetylcysteine, to augment cellular antioxidant defence *in vitro*. *Respir. Med.*, **1997**, *91*, 159-168.
- [71] Mendiratta, S.; Qu, Z.C.; May, J.M. Erythrocyte ascorbate recycling: antioxidant effects in blood. *Free Radic. Biol. Med.*, **1998**, *24*, 789-797.
- [72] Dickinson, D.A.; Forman, H.J. Cellular glutathione and thiols metabolism. *Biochem. Pharmacol.*, **2002**, *64*, 1019-1026.
- [73] Wilson, J.X. The physiological role of dehydroascorbic acid. *FEBS Letters*, **2002**, *527*, 5-9.
- [74] Doroshov, J.H. Glutathione peroxidase and oxidative stress. *Toxicol. Lett.*, **1995**, *82/83*, 395-398.
- [75] Tacchini, L.; Pogliaghi, G.; Radice, L.; Bernelli-Zazzera, A.; Cairo, G. Post-transcriptional control of increased hepatic catalase gene expression in response to oxidative stress. *Redox Rep.*, **1996**, *2*, 273-278.
- [76] Wang, P.; Chen, H.; Qin, H.; Sankarapandi, S.; Becher, M.W.; Wong, P.C.; Zweier, J.L. Overexpression of human copper, zinc-superoxide dismutase (SOD1) prevents postischemic injury. *Proc. Natl. Acad. Sci. USA*, **1998**, *95*, 4556-4560.
- [77] Chen, Z.; Oberley, T.D.; Ho, Y.; Chua, C.C.; Siu, B.; Hamdy, R.C.; Epstein, C.J.; Chua, B.H. Overexpression of CuZnSOD in coronary vascular cells attenuates myocardial ischemia/reperfusion injury. *Free Rad. Biol. Med.*, **2000**, *29*, 589-596.
- [78] Csonka, C.; Pataki, T.; Kovacs, P.; Müller, S.L.; Schroeter, M.L.; Tosaki, A.; Blasig, I.E. Effects of oxidative stress on the expression of antioxidative defense enzymes in spontaneously hypertensive rat hearts. *Free Radic. Biol. Med.*, **2000**, *29*, 612-619.
- [79] Bocci, V. Does ozone therapy normalize the cellular redox balance? *Med. Hypotheses*, **1996**, *46*, 150-154.
- [80] León, O.S.; Menéndez, S.; Merino, N.; Castillo, R.; Sam, S.; Pérez, L.; Cruz, E.; Bocci, V. Ozone oxidative preconditioning: a protection against cellular damage by free radicals. *Mediators Inflamm.*, **1998**, *7*, 289-294.
- [81] Larini, A.; Bianchi, L.; Bocci, V. The ozone tolerance: I) Enhancement of antioxidant enzymes is ozone dose-dependent in Jurkat cells. *Free Radic. Res.*, **2003**, *37*, 1163-1168.
- [82] Bocci, V. Scientific and medical aspects of ozone therapy. State of the art. *Arch. Med. Res.*, **2006**, *37*, 425-435.
- [83] Bocci, V. Is it true that ozone is always toxic? The end of a dogma. *Toxicol. Appl. Pharmacol.*, **2006**, *216*, 493-504.
- [84] Bocci, V.A. Why orthodox medicine has not yet taken advantage of ozone therapy. *Arch. Med. Res.*, **2008**, *39*, 259-260.
- [85] Bocci, V.; Aldinucci, C.; Mosci, F.; Carraro, F.; Valacchi, G. Ozonation of human blood induces a remarkable upregulation of heme oxygenase-1 and heat stress protein-70. *Mediators Inflamm.*, **2007**, 26785.
- [86] Elbirt, K.K.; Bonkovsky, H.L. Heme oxygenase: recent advances in understanding its regulation and role. *Proc. Assoc. Am. Physicians*, **1999**, *111*, 438-447.
- [87] Snyder, S.H.; Barañano, D.E. Heme oxygenase: a font of multiple messengers. *Neuropsychopharmacology*, **2001**, *25*, 294-298.
- [88] Zuckerbraun, B.S.; Billiar, T.R. Heme oxygenase-1: a cellular Hercules. *Hepatology*, **2003**, *37*, 742-744.
- [89] Abraham, N.G.; Kappas, A. Pharmacological and clinical aspects of heme oxygenase. *Pharmacol. Rev.*, **2008**, *60*, 79-127.
- [90] Bocci, V. In *Oxygen-ozone therapy. A critical evaluation*, Bocci, Ed.; Kluwer Academic Publisher: Dordrecht, **2002**; chapter 22, pp. 233-240.
- [91] Goldman, M. Cancer risk of low-level exposure. *Science*, **1996**, *271*, 1821-1822.
- [92] Calabrese, E.J. Paradigm lost, paradigm found: the re-emergence of hormesis as a fundamental dose response model in the toxicological sciences. *Environ. Pollut.*, **2005**, *138*, 379-411.
- [93] Jacobs, M.T. Untersuchung über zwischenfälle und typische komplikationen in der ozon-sauerstoff-therapie. *OzonNachrichten*, **1982**, *5*, 1-5.
- [94] Travagli, V.; Zanardi, I.; Silviotti, A.; Bocci, V. A physicochemical investigation on the effects of ozone on blood. *Int. J. Biol. Macromol.*, **2007**, *41*, 504-511.
- [95] Bocci, V. The case for oxygen-ozonotherapy. *Br. J. Biomed. Sci.*, **2007**, *64*, 44-49.
- [96] Rokitanski, O.; Rokitanski, A.; Steriner, J.; Trubel, W.; Viebahn, R.; Washüttl, J. Die ozontherapie bei peripheren, arteriellen durchblutungsstörungen; klinik, biochemische und blutgasanalytische untersuchungen. In : Wasser IOA, Ozon-Weltkongress, Berlin **1981**. p. 53-75.
- [97] Matassi, R.; D'Angelo, F.; Bisetti, P.; Colombo, R.; Vaghi, M. Terapia con ozono per via parenterale nelle arteriopatie obliteranti periferiche: meccanismo biochimico e risultati clinici. *Il Giornale di Chirurgia*, **1987**, *VIII*, 109-111.
- [98] Giunta, R.; Coppola, A.; Luongo, C.; Sammartino, A.; Guastafierro, S.; Grassia, A.; Giunta, L.; Mascolo, L.; Tirelli, A.; Coppola, L. Ozonized autohemotransfusion improves hemorheological parameters and oxygen delivery to tissues in patients with peripheral occlusive arterial disease. *Ann. Hematol.*, **2001**, *80*, 745-748.
- [99] Tylicki, L.; Niewegłowski, T.; Biedunkiewicz, B.; Burakowski, S.; Rutkowski, B. Beneficial clinical effects of ozonated autohemotherapy in chronically dialysed patients with atherosclerotic ischemia of the lower limbs-pilot study. *Int. J. Artif. Organs.*, **2001**, *24*, 79-82.
- [100] Tylicki, L.; Niewegłowski, T.; Biedunkiewicz, B.; Chamienia, A.; Dębska-Slizien, A.; Aleksandrowicz, E.; Lysiak-Szydłowska, W.; Rutkowski, B. The influence of ozonated autohemotherapy on oxidative stress in hemodialyzed patients with atherosclerotic ischemia of lower limbs. *Int. J. Artif. Organs*, **2003**, *26*, 297-303.
- [101] Clavo, B.; Pérez, J.L.; López, L.; Suárez, G.; Lloret, M.; Rodríguez, V.; Macías, D.; Santana, M.; Morera, J.; Fiuza, D.; Robaina, F.; Günderoth, M. Effect of ozone therapy on muscle oxygenation. *J. Altern. Complement. Med.*, **2003**, *9*, 251-256.
- [102] Clavo, B.; Catalá, L.; Pérez, J.L.; Rodríguez, V.; Robaina, F. Ozone Therapy on Cerebral Blood Flow: A Preliminary Report. *Evid. Based Complement. Alternat. Med.*, **2004**, *1*, 315-319.
- [103] Tylicki, L.; Biedunkiewicz, B.; Niewegłowski, T.; Chamienia, A.; Slizien, A.D.; Luty, J.; Lysiak-Szydłowska, W.; Rutkowski, B. Ozonated autohemotherapy in patients on maintenance hemodialysis: influence on lipid profile and endothelium. *Artif. Organs*, **2004**, *28*, 234-247.
- [104] Biedunkiewicz, B.; Tylicki, L.; Niewegłowski, T.; Burakowski, S.; Rutkowski, B. Clinical efficacy of ozonated autohemotherapy in hemodialyzed patients with intermittent claudication: an oxygen-controlled study. *Int. J. Artif. Organs*, **2004**, *27*, 29-34.
- [105] De Monte, A.; van der Zee, H.; Bocci, V. Major ozonated autohemotherapy in chronic limb ischemia with ulcerations. *J. Altern. Complement. Med.*, **2005**, *11*, 363-367.
- [106] Merin, O.; Attias, E.; Elstein, D.; Schwalb, H.; Bitran, D.; Zimran, A.; Silberman, S. Ozone administration reduces reperfusion injury in an isolated rat heart model. *J. Card. Surg.*, **2007**, *22*, 339-342.

8 *Cardiovascular & Haematological Disorders-Drug Targets*, 2009, Vol. 9, No. 2

Bocci et al.

- [107] Wasser, G.H. In *Ozon-Handbuch. Grundlagen. Prävention. Therapie*; Beck and Viebahn-Hänsler, Eds.; Ecomed: Landsberg, **1995**; V-6.3, pp. 1-12.
- [108] Olin, J.W. A multicenter, randomized, double-blind, placebo-controlled study of immune modulation therapy in patients with symptomatic peripheral arterial disease: the SIMPADICO Trial. American College of Cardiology 55th Annual Scientific Sessions; March 11–14, **2006**, Atlanta, GA. Late-Breaking Clinical Trials I.
- [109] Sliwa, K.; Ansari, A.A. Immunosuppression as therapy for congestive heart failure. *Lancet*, **2008**, *371*, 184-186.
- [110] Fildes, J.E.; Shaw, S.M.; Yonan, N.; Williams, S.G. Non-specific immunomodulation in chronic heart failure. *Lancet*, **2008**, *37*, 2083.
- [111] Bocci, V. Non-specific immunomodulation in chronic heart failure. *Lancet*, **2008**, *37*, 2083.
- [112] Bocci, V. The failure of the ACCLAIM trial is due to an irrational technology. *Int. J. Cardiol.*, **2009**, doi:10.1016/j.ijcard.2008.10.001, in press.

Received: 13 February, 2008

Revised: 11 March, 2009

Accepted: 12 March, 2009

Current Concepts of Oxygen Ozone Therapy for Dentistry in the United States

J.A. ROTHCHILD¹, R.E. HARRIS², P.J. MOLLICA³

¹ School of Integrative Biologic Dental Medicine, Saddle Brook, NJ; Rush University Medical School, Chicago, IL; Capital University of Integrative Medicine, Washington, DC; Private practice, Durango, CO and Hoffman Estates, IL, USA.

² Integrative Biologic Dentistry, School of Integrative Biologic Dental Medicine, Saddle Brook, NJ; Capital University of Integrative Medicine, Washington, DC. Private practice, Louisville, KY, USA

³ Integrative Biologic Dentistry, School of Integrative Biologic Dental Medicine, Saddle Brook, NJ; Capital University of Integrative Medicine, Washington, DC; Oro-Facial Pain, Hackensack University Medical Center, Hackensack, NJ. Private practice, Saddle Brook, NJ, USA

Key words: oxygen ozone therapy, dentistry, United States

Introduction

Oxygen ozone therapy (OOT) has been used successfully in many countries throughout the world for many years. It has been recently gaining interest in the medical community in the wake of an increasing number of scientific and clinical papers published in international peer reviewed medical journals. Many of the basic mechanisms of the action of ozone both *ex vivo* and *in vivo* are now well understood. The method by which ozone has affected the modulation of interleukin production and additional biological pathways has been adequately explained by multiple researchers^{1,2}. This research has resulted in the rationale for the use of ozone in many pathological conditions related to pain, inflammation, oxidative stress, cancer, atherosclerosis, diabetes, acute infections and chronic infections³.

Conventional dentistry in the United States has been very slow to embrace OOT in part because it had not been included in the didactic or clinical curriculum in any School of Dentistry in the United States. However, in December of 2008 the first presentation on "Ozone in Dental Medicine" was delivered at Tufts School of Dentistry by Dr. Philip Mollica. Prior to this presentation, the institutions had not been receptive to investigating the possible research opportunities that would verify the previous favorable findings from institutions in Germany, Great Britain, Italy, Spain, Brazil, Cuba, Russia, etc.

The recent movement to use OOT in the United States was initiated by a small group of dentists. They attended a demonstration and participation class using OOT at Capital University of Integrative Medicine (a post doctoral program

in integrative health) in 1998. Soon after that presentation, a group of eight dentists from that class decided to pursue further research into the possible benefits of OOT.

The group obtained the corona discharge type of medical ozone units that utilize medical oxygen as the oxygen source and began limited clinical trials in April 2000.

After successful clinical trial results, an IRB approved study was granted by Capital University of Integrative Medicine to further research and define the uses of OOT. The primary researchers, Dr. Philip Mollica and Dr. Robert Harris, decided to utilize the evidence-based research model and establish sub-investigators in a practice-based research network. This model was successful and was repeated by the current research institution, The American College of Integrative Medicine and Dentistry. To satisfy the requests of practicing dentists, Dr. Mollica and Dr. Harris, then began offering a seminar series to teach dentists the theory and clinical applications for infection control utilizing OOT. To date, they have taught OOT to over 2,000 dentists through their seminars and presentations at major meetings, both nationally and internationally.

Clinical research is currently being conducted and monitored from multiple practice-based research network clinical centers throughout the United States⁴. As a result of the research findings, foundational protocols have been developed to address common oral infections such as periodontal disease, endodontic infections, dental caries, osteomyelitis, bisphosphonate induced osteonecrosis, stomatitis and herpetic lesions. Additional therapeutic protocols have been developed for tooth related sinus infections, neuralgia, pulpal hyper-

sensitivity, extractions, and temporomandibular joint (TMJ) symptomology.

The International Academy of Oral Medicine and Toxicology has endorsed OOT in dentistry as scientifically valid and has published a brochure to explain the value of the therapy to dentists and patients.

Therapeutic Modalities

Therapeutic methods of administration of intraoral Oxygen-Ozone (OO) include: injection of gas, irrigation with ozonated water, insufflation of periodontal pockets with gas, and topical application of ozonated oil. Injection protocols include intraosseous injection of the OO gas mixture, locally into the alveolus, subgingivally, intramuscularly, inferior alveolar nerve area, and into the pterygoid space area. These injection protocols are for treatment of all types of oral infections. Irrigation with ozonated water is also utilized for oral infections including stomatitis, herpetic lesions and periodontal infections (subgingival). Insufflation techniques with OO gas are generally utilized for caries, periodontal infections and endodontic treatment. In addition, a technique utilizing silicone full arch trays has been developed for treatment of periodontal disease, caries and bisphosphonate osteonecrosis. Extraoral therapeutic protocols include nasal and ear insufflation, temporomandibular joint injections, trigger point injections and craniofacial lymphatic injections. These modalities are used for both primary and secondary supportive treatment techniques.

OO Treatment Goals in Dentistry

The therapeutic goals that support established standard of care procedures are as follows:

- Elimination of pathogens
- Restoration of proper oxygen metabolism
- Induction of friendly ecologic environment
- Increased circulation
- Immune modulation
- Stimulation of the humoral anti-oxidant system

Treatment of Dental Caries and Operative Dentistry

More than 30 studies⁵ have been presented showing that low concentrations of OO gas causes inhibition of pit and fissure caries, root surface caries and interproximal carious lesions. These same studies have also shown that reversal of decay in

carious lesions occurs with exposure to OO in as little as ten seconds. The protocols developed by the American College of Integrative Medicine and Dentistry encompass utilization of OO for procedures including: pit and fissure sealants, caries removal with subsequent restoration, dentinal hypersensitivity, crown and bridge preparation, carious exposures, etc. The procedure is to isolate the tooth or preparation and flow the gas slowly into the area to be treated for 45-60 seconds. This procedure will locally kill the microorganisms which are present in the tooth structure. The use of proper evacuation technique is essential to avoid inhalation of the gas. If inhaled, the oxidant nature of the ozone can cause an irritation to the eyes and the mucosal lining of the respiratory tract because these tissues have very limited anti-oxidant capacity.

Treatment of Root Canals

Endodontic (root canal) treatment of infected teeth has long been a treatment of choice and the standard of care in dentistry for an infected tooth. Endodontic treatment involves cleaning out the main canal(s) of a tooth with instrumentation, irrigation and chemicals (sodium hypochlorite-bleach). These canal(s) are then filled with a material called gutta percha before the tooth is finally restored with a crown. This procedure is intended to sterilize the tooth from all the invading bacteria that caused the tooth and the surrounding bone to become infected.

The classical endodontic community feels that this procedure prevents any bacteria from living within the tooth or ever invading the tooth again from the alveolar bone, thus saving the tooth. This is a contested theory that has been disputed by the allopathic and the integrative medical/dental communities. Studies have shown that following endodontic therapy, some bacteria, fungi and viruses remain in the multitude of the very small lateral canals and dentinal tubules that transverse the tooth root and communicate with the periodontal tissue. These studies have shown that the obligate anaerobes (which can include bacteria, virus and fungi) can remain within these canals and are even found all the way to the cementoenamel junction.

A Japanese study published in 2004⁶ demonstrated that the use of ozonated water had the same antimicrobial activity as 2.5 percent sodium hypochlorite without the tissue toxicity. The study also showed that following ozone therapy there was high metabolic activity of the associated fibroblasts indicating an increase in the healing

process. A Brazilian study, performed on dogs, found that the use of ozonated oil was actually slightly more effective than calcium hydroxide as an intracanal medicament for the treatment of *enterococcus faecalis* infections. The anaerobic bacteria create an infection that results in an area that is acidic with positively charged suppurative fluids. OO gas is the third strongest oxidant. It carries a negative charge and is electrochemically attracted to the positive charge of the infected environment. This results in the death of the pathogens and disinfection of the area.

Standard of care endodontic procedures are employed during diagnostics and treatment. Then OOT is used for disinfection of the root canals and dentinal tubules. The following steps should be added before the final fill of the canal(s):

- The files are coated with ozonated olive oil for lubrication and disinfection.
- The canals are prepared and then irrigated with ozonated water and dried.
- Before placing the root canal filling, the canals are provided with a slow insufflation of gas (45-60 seconds) with an ozone concentration of 45-50 mcg/ml.

The insufflation process allows the OO mixture to travel electrochemically into the lateral canals and tubules killing the positively charged microbes by oxidizing their cell membranes.

Treatment of Periodontal Disease

Periodontal disease is a multifactorial disease process in the mouth. It has been linked systemically to other diseases such as atherosclerosis, bronchitis, diabetes, preterm and low weight births, pancreatic cancer and others.

Traditional treatment has been either conservative treatment by root planing and scaling, surgical intervention with a scalpel or LASER therapy (for example the LANAP procedure with the Periolas, an Nd: YAG LASER).

In cases where treatment is by root planing and scaling, the sulci and pockets are initially irrigated with ozonated water by use of a syringe and a maxiprobe type canula. This process will reduce the initial pathogenic load on the patient, both locally and systemically prior to the root planing and scaling procedures. After treatment of a quadrant or half the mouth, each pocket and sulcus is insufflated with OO gas. The gas goes directly into the crevicular fluid and the tissues and sterilizes the area, thus eliminating the pathogenic organisms.

For patients undergoing LASER therapy with the Periolas, it is recommended that ozonated water be used during ultrasonic debridement.

For certain cases, the silicone tray isolation technique may be utilized. This involves fabrication of appliances made of silicone that fits snugly onto each arch. Each appliance has an inlet and an outlet valve. A low/medium concentration of OO gas flows continuously through the appliance saturating the teeth and periodontium with OO gas. Ozone gas is introduced into the tray through the inlet port of the tray. The small suction evacuator is attached to the outlet valve allowing the excess gas to be vacuumed away to prevent inhalation of the gas. This treatment requires multiple visits. Routine recall treatment for minor cases, such as gingivitis, utilizes pretreatment rinsing with ozonated water, irrigation of the periodontium and insufflation of any periodontal pockets. In all cases, the patient is given a jar of ozonated olive oil to take home with them and apply topically to the soft tissue. This will insure a continuous dose of OO in the form of ozone, to the tissues. It also continues to eliminate the microbes that create the biofilm that causes reinfection of the surrounding tissues.

Adjunct Therapy for Extractions, Other Surgical Procedures and Biphosphonate Osteonecrosis

OO is so versatile that it can be used for almost any type of dental procedure. After a tooth is extracted or any basic surgical procedure it is recommended post-surgically to irrigate and insufflate the area. This reduces the positive electric potential of the wound and potential scarring with the negatively charged gas or water. Healing of the wound is generally much faster, with little or no complications. Biphosphonate necrosis has been extremely difficult to treat medically and surgically. There has been some success with OO utilizing the foundational protocols along with intraosseous injections and intraoral silicone tray treatment of the osteonecrotic lesion. The patient is always sent home with a jar of ozonated olive oil as a postoperative dressing for the wound.

Extraoral Techniques

Part of the foundational protocol involves ear insufflation and nasal insufflation with low concentrations of OO. Ear insufflation is a technique to deliver the OO into the external, middle and inner ear. The tympanic membrane is vascularized and some ozone can enter the bloodstream by this route. Ozone being a potent oxidant easily perfuses into the blood and reacts immediately with a number of molecules present in the fluids such as antioxidants, proteins, carbohydrates, and

polyunsaturated fatty acids³. OO, when administered nasally has to be bubbled through olive oil. This produces triozonides of triolein, which are not oxidants. But when the ozonides are inhaled they have therapeutic effects similar to ozone. The foundational protocols of the American College of Integrative Medicine and Dentistry for ozone therapy also include extraoral injections of small concentrations of OO. These small amounts, usually 1.0 ml per site, are infiltrated either subdermally or subcutaneously along the path of the external jugular chain of lymph nodes, the cervical lymph node system, the thyroid lymph nodes, the right thoracic duct and the left thoracic duct.

Conclusion

Ozone is the perfect substance for use in dental procedures.

It disinfects the tissues treated and leaves no toxic residues like chlorinated products.

It performs this task by oxidizing the cell membranes of pathogenic organisms and killing them.

The oxidizing effect of ozone is as follows: it requires one molecule of ozone to kill the same number of bacteria that would require 3,000-10,000 molecules of chlorine for the same effect and ozone performs this kill 3500 times faster than chlorine⁴.

References

- 1 Lamberto Re. Medici & Medici, Periodico Trimestrale Ordine Medici-Chirurghi e Odontoiatri della Provincia di Ancona, N. 16 Luglio 2008; 18-20.
- 2 Wentworth, P. Jr., Wentworth AD, Zhu X, et al., Proc Natl Acad Sci. 2003 Feb. 18; 100(4): 1490-3, Epub, Feb. 7, 2003.
- 3 V. Bocci. Ozone, A New Medical Drug, Springer Publishing; 2005.
- 4 Mollica P, Harris R. Dental Economics. September 2007.
- 5 E. Lynch, et al. Ozone: The Revolution in Dentistry. Quintessence; 2004.
- 6 Nagayoshi, M., Kitamura C, Fukuzumi T, et al. Antimicrobial effect of ozonated water on bacteria invading dentinal tubules. J Endod. 2004; Nov. 30 (11): 778-81.
- 7 Vierira Silveira AM, LopesI HP, Siqueira JF, Jr. Periradicular repair after two-visit endodontic treatment using two different intracanal medications compared to single-visit endodontic treatment. Braz Dent J. 2007; 18 (4): 299-304.

John A. Rothchild, MD
DDS, 555 Rivergate Lane Suite, B1-106
Durango, CO 81301, USA
E-mail: jrothchild@aol.com

Ozone and Ozonated Oils in Skin Diseases: A Review

V. TRAVAGLI¹, I. ZANARDI¹, G. VALACCHI^{2,3}, V. BOCCI⁴

¹ Dipartimento Farmaco Chimico Tecnologico, Università degli Studi di Siena, Viale Aldo Moro 2, 53100 Siena, Italy

² Dipartimento di Scienze Biomediche, Università degli Studi di Siena Viale Aldo Moro 2, 53100 Siena, Italy

³ Department of Food and Nutrition, Kyung Hee University, Seoul 130-761, Republic of Korea

⁴ Dipartimento di Fisiologia, Università degli Studi di Siena, Viale Aldo Moro 2, 53100 Siena, Italy

Key words: ozone, ozonated, oils, skin

ABSTRACT - Although orthodox medicine has provided a variety of topical anti-infective agents, some of them have become scarcely effective owing to antibiotic- and chemotherapeutic-resistant pathogens. For more than a century, ozone has been known to be an excellent disinfectant that nevertheless had to be used with caution for its oxidizing properties. Only during the last decade it has been learned how to tame its great reactivity by precisely dosing its concentration and permanently incorporating the gas into triglycerides where gaseous ozone chemically reacts with unsaturated substrates leading to therapeutically active ozonated derivatives. Today the stability and efficacy of the ozonated oils have been already demonstrated, but owing to a plethora of commercial products, the present paper aims to analyze these derivatives suggesting the strategy to obtain products with the best characteristics.

1. Introduction

The increase of ageing, obesity, and diabetes in conjunction with inappropriate healthcare programs have emphasized the problem of having to treat almost 1.5 billion people affected by skin and mucosal infections due to bacteria, viruses, protozoa, and dysmetabolism. Pathologies range from the diabetic foot (ulcer with necrosis), bed sores, ulcers after a trauma or burns, chronic viral infections due to either herpes virus I and II, or human papilloma viruses, vaginal infections now frequent also in young girls due to *Candida*, *Trichomonas*, and *Chlamidia*, rectal mucosa infections such as anal ragadis, abscesses with fistula to end with mouth aphthous ulcers. These infections are rarely deadly but are considerably distressing because many patients often suffer of diabetes or vascular diseases with tissue hypoxia, other patients are immunosuppressed drug addicts, or with concomitant HIV infection. Official medicine provides a variety of drugs that are expensive and often poorly efficacious because

infections in hypoxic tissue contain methicillin-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Patients are suffering not only because they become uncompliant to frequent medications but they are discouraged by observing a lack of healing¹. Wound healing is a multiphase process involving blood clotting, inflammation, tissue proliferation, and remodelling², but both innate and adoptive immune systems are too often hindered by the chronic infection naturally difficult to overcome. This is also the reason explaining the failure of growth factors in heavily contaminated ulcers^{3,4}.

The judicious use of ozone (O₃) appears providential because first of all eliminates the pathogens and then, by releasing oxygen (O₂), activates the proliferation of fibroblasts, hence the building of intercellular matrix with consequent proliferation of keratinoblasts and successive healing.

In Section 2, we propose to briefly review the physical chemistry of oil ozonation and all the basic analyses necessary for demonstrating the quality of the obtained products. In Section 3, it appears useful to inform readers that both skin

and mucosae are sensitive to excessive amounts of gaseous O_3 as there are clear demonstrations of a variety of alterations linked to a prolonged exposure. In Section 4, we will then clarify the various procedures devised to enhance the disinfectant and healing-promoting properties of O_3 . Finally, after an extensive analysis of a cornucopia of proposals, we will try to suggest guidelines for the future medical application of topical ozone and its derivatives (Section 5).

2. Physical Chemistry of Oil Ozonation with a Description of the Analytical Methods for Characterizing the Process

Unsaturated lipid substrates react with insufflated gaseous O_2/O_3 mixture leading to therapeutically active ozonated derivatives (Figure 1).

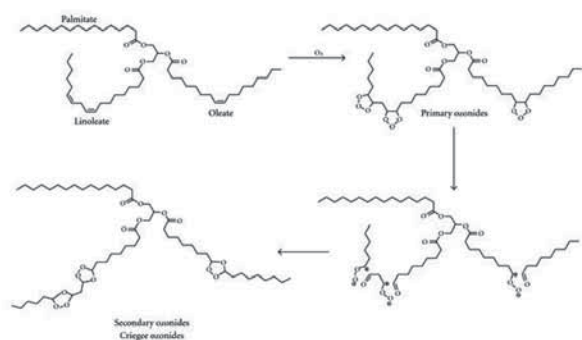


Figure 1 Representative chemical structures of ozonated derivatives which are formed by chemical reaction of ozone with unsaturated triglycerides. The primary ozonides are transient, unstable species which rearrange in the normal, secondary ozonides also known as Criegee ozonides.

Briefly, the postulated mechanism known as Criegee reaction provides that ozone combines with an unsaturated bond to form an initial, unstable primary ozonide which readily decomposes to form a zwitterions and a carbonyl fragment. In anhydrous environment these substrates combine to give the typical cyclic trioxolane derivative.

However, the word “ozonated” is itself without scientific meaning if it is not associated with “how much” peroxides are present in the oil. In fact, from a therapeutic point of view, the ozonide compositions have the capacity to deliver active O_2 and/or other useful species deep within the lesion without causing primary skin irritation. The few studies concerned with the therapeutic effects of ozonated oils on acute cutaneous wound healing in animal models do not investigate the dose/behaviour response, expressed as the amount of peroxides existing in the ozonated derivative used⁵. Recently, a quantitative evaluation of the therapeutic effect of topically applied ozonated sesame oil on acute

cutaneous wound healing in mice as animal model has been developed⁶. The results indicate that both low (<1000) and high doses (>3000), as expressed in terms of peroxide value (see the corresponding section in this paper), delay cutaneous wound healing. Such an evidence is reinforced by a number of results between groups where the “middle” concentration (about 1500) has the most beneficial effect in accelerating the wound closure ratio.

From an industrial applicative viewpoint, the overall quality of ozonated derivatives depends upon several parameters, such as: (i) the type and the quality of ozone generators; (ii) the ozonation conditions, in terms of reactors and time, material type and amount, presence of water and/or catalyzers; (iii) the efficacy of the ozonizer, in terms of O_3 concentration output, gas flow, gas carrier. As for the latter, the use of medical grade O_2 instead of air is an important point to be considered; in fact, air feedstock (containing about 78% of nitrogen) used for the ozonation of unsaturated substrates could lead to the production of potentially toxic nitrated by-products⁷, and to a significant decrease of the ozonation efficiency⁸. Another important feature is that ozonated oil has to be unequivocally characterized in terms of the species contents as well as the reaction kinetics. For these purposes, the knowledge of the physicochemical properties of ozonated vegetable oils during production has a great importance for their characterization and identification. For determining the quality of ozonated products, spectroscopic techniques, as Fourier-Transformed Infrared (FT-IR) and 1H and ^{13}C -NMR⁹, together with analytical methods as peroxide, acidity, and iodine values as well as viscometric determination are usually carried out¹⁰.

2.1. FT-IR Spectroscopy

FT-IR spectroscopy is used to highlight differences in the functional groups during the oil ozonation, in particular the decrease of the bands corresponding to both $C=C$ and $=C-H$ stretching (e.g., sesame oil at 1654 cm^{-1} and 3009 cm^{-1} , resp), and the increase of the band corresponding to ozonide CO stretching (e.g., sesame oil at 1105 cm^{-1}).

Ozonated samples can be analyzed using two different methods.

(1) An adequate aliquot (usually about $2\ \mu\text{L}$) of sample is deposited between two disks of KBr , avoiding air bubble formation, then the percentage transmittance or other suitable parameters are measured in the range $4000\text{--}800\text{ cm}^{-1}$. Spectra are obtained setting the appropriate scan summations and minimal resolution (generally, 16 at 4 cm^{-1} , resp.).

(2) An adequate aliquot (usually about $2\ \mu\text{L}$) of sample is dissolved in a suitable solvent (preferably chloroform) and then the solution is settled in

the sample holder avoiding air bubble formation, then the transmittance (expressed as a percentage) or other suitable parameters are measured in the range 4000–800 cm^{-1} . Spectra are obtained setting the appropriate scan summations and minimal resolution (generally, 16 at 4 cm^{-1} , resp.).

2.2. NMR Spectroscopy

^1H and ^{13}C NMR spectroscopies are performed to obtain more information about the variation of the functional groups involved in the reaction of ozonation. Both the disappearance of the signals relative to protons and carbons on the double bond (e.g., in sesame oil 5.29 ppm, and various signals in the range 127.8–130.0 ppm, resp.) and the parallel appearance of a signal on the proton and carbon of 1,2,4-trioxolane (e.g., in sesame oil in the 5.11–5.08 ppm range, and 103.4–104.3 ppm range, resp.) are evidenced. Quantitative analysis can be performed by spectra normalized with respect to the integral areas of the OCH_2 protons (glycerol) that remain constant during the whole process.

Spectra will be obtained using suitable instruments by solubilizing the ozonated sample in a proper solvent (preferably CDCl_3). Particularly, an adequate aliquot (usually about 100 μL) of sample is solubilised with 750 μL of CDCl_3 in a 5 mm NMR tube, then the analysis will be performed. To obtain quantitative data, it is sufficient to perform a ^1H -NMR, while ^{13}C -NMR essentially provides qualitative informations⁹.

2.3. Iodine Value

The iodine value (IV) represents the quantity of iodine (in grams) that will react with the double bonds in 100 grams of sample. IV is determined according to the Pharmacopoeia monographs. The IV is calculated by means of the following equation:

$$\text{IV} = \frac{1.269 \cdot (n_1 - n_2)}{m}, \quad (1)$$

where n_1 is the volume in mL of thiosulphate solution (0.1M) used for carry out a blank test, n_2 is the volume in mL of thiosulphate solution (0.1M) used for the titration and m the quantity, in grams, of substance. It is, therefore, a measure of the total number of double bonds present in the sample and for such a reason it is a chemical analysis useful for evaluating the decrease of double bonds during the oil ozonation process, giving information about the 1,2,4-trioxolane formation.

2.4. Acid Value

The acid value (AV) is an index that expresses, in mg, the quantity of potassium hydroxide required to neutralise the free acids presents in 1g of the substance.

The AV is calculated by means of the following equation:

$$\text{AV} = \frac{5.610 \cdot n}{m}, \quad (2)$$

where n is the volume in mL of titrant and m the quantity, in grams, of substance.

It is representative of the acidity level of the product and it represents an index of the degradation by-products that could be formed during the ozonation process.

2.5. Peroxide Value

Peroxide value, (PV), is usually used as an indicator of the advancement and/or the control of the ozonation process because of its simplicity, rapidity, and low cost. Moreover, the PV may be adequate for the stability evaluation of vegetable oil ozonides and it appears to be very important for commercial distribution as well as for the determination of the better storage modalities. However, it had been necessary to standardize the methodology for a validated PV.

In the present paper, a detailed analysis of PV assessments of ozonated lipid derivatives based on both literature data and our laboratory experiments will be presented together with their possible correlations with other techniques. Such a report allows an in-depth acquaintance of the ozonation process of vegetable oils as well as of the related products obtained, allowing to define the quality parameters useful for industrial purposes. Specifically, the peroxide value (PV) represents the quantity of peroxide expressing in milliequivalents of active O_2 contained in 1000 g of the sample.

For the PV evaluation, three different methods were adopted.

(a) First official monograph described in Pharmacopoeia (e.g., European Pharmacopoeia, British Pharmacopoeia, United States Pharmacopoeia), which provides the solubilization of sample in 30 mL of chloroform/glacial acetic acid (2:3), the addition of saturated potassium iodide solution (0.5 mL) and the titration after 1 minute with a solution of sodium thiosulphate.

(b) Second method described by Martinez Tellez et al.¹¹, which always provides the solubilization of sample in 30 mL of chloroform/glacial acetic acid (2:3) and the addition of saturated potassium iodide solution (0.5 mL), but the titration is done after 24 hours.

(c) Third method recently proposed¹⁰. Briefly, 2 g of SO were weighed in a 250 mL conical flask and 30 mL of chloroform/glacial acetic acid (2:3) were added. Then, 3.0 mL of saturated potassium iodide solution were added. The flask was stirred at reflux temperature (60°C) for various times (5–180 minutes). After this time, the solution was

cooled and 25 mL of water were added. Solutions of sodium thiosulphate at the appropriate concentration (0.0001–0.1 M) were used for the titration.

In all determinations the PV was calculated by means of the following equation:

$$PV = \frac{1000 \cdot (V_1 - V_0) \cdot c}{m}, \quad (3)$$

where V_1 is the volume in mL of thiosulphate solution used for the titration, V_0 is the volume in mL of thiosulphate solution used for carry out a blank, c the thiosulphate concentration and m the sample quantity (grams).

The ozonation efficiency (expressed as a percentage) represents ratio of the amount of peroxidation due to ozonation process, as estimated by PV value, to the O_3 total amount applied to the system. It was calculated by means of the following equation:

$$OE = \frac{(PV_s - PV_0)}{1000} \times \frac{24}{OAD} \times 100, \quad (4)$$

where PV_s is the ozonated sample PV, PV_0 is the PV of untreated sample, and OAD stands for the applied dose (mg/g).

2.6. Viscosity Measurement

Viscosity evaluation is a useful technique because it is fast and it could be online, giving an estimation of the double bonds present in the sample. In fact, the greater the ozonation time the higher the product viscosity because of the disappearance of the double bonds. Moreover, its typical trend can be a useful tool in providing a rapid quality control assessment during the entire ozonation process, as well as to decide on the process time for obtaining the desired ozonation level of the sample⁹.

3. Cutaneous Responses to Environmental Ozone Exposure

The skin, along with the respiratory tract, is directly exposed to environmental pollutants including O_3 , an important constituent of photochemical smog. Although numerous studies have documented effects of O_3 on the respiratory tract in animals and humans¹²⁻¹⁵, only recently some studies characterizing its effect on cutaneous tissue have been published¹⁶⁻²⁰.

The skin consists of two main layers, the inner dermis, mainly composed of fibroblasts and connective tissue matrix, and the outer epidermis, which contains keratinocytes that, by progressively differentiating to form enucleate corneocytes, become imbedded in a lipid matrix and together comprise the outermost part of the epidermis, the stratum corneum (SC)^{21,22}.

Previous studies have shown that exposure to O_3 results in the depletion of both water soluble and lipophilic antioxidants such as uric acid, ascorbic acid, and tocopherol, and this was accompanied by increase in parameters of both lipid peroxidation and protein modification, primarily in the outermost skin layers^{16,17,23}.

In further studies, we were also able to show that the exposure of hairless mice to O_3 will not only deplete the antioxidant levels and increase oxidative markers but these molecules are able to induce active cell responses.

These effects can be briefly summarized as follows.

(1) Induction of Redox Sensitive Transcription Factors

Ozone, like many others environmental challenges, is able to activate transcriptional factors redox sensitive such as Nuclear Factor κ B (NF κ B). This transcriptional factor acts as an activator for a multitude of proinflammatory genes (IL-8, TNF α , TGF β) and adhesion molecules (ICAM and VCAM). It has been assessed that O_3 is able to activate NF κ B using both *in vitro* and *in vivo* systems. Thiele et al.¹⁶, using an immortalized human keratinocytes (HaCaT cells), were able to show that O_3 induced the activation of NF κ B by electrophoretic mobility shift assay (EMSA). Ozone induced a dose dependent activation of the transcription factor.

This effect was likely to be mediated by ROS, particularly H_2O_2 , because it was inhibited by the incubation of the cells with lipid soluble antioxidants (tocopherol).

(2) Induction of Heat Shock Protein (HSP) and Inflammatory Markers

As a consequence of the induction of transcription factors, O_3 exposure (6 days to 0.8 μ g/mL for 6 hours/day) induced the expression of proinflammatory markers in skin homogenates such as cyclooxygenase-2 (COX-2). This induction was accompanied by an increase level of heat shock protein (HSP) 32, also known as heme oxygenase-1 (HO-1). In this paper, we were the first to demonstrate the upregulation of HSPs 27, 32 and 70 in homogenized murine skin upon O_3 exposure. HSP27 showed the earliest (2 hours) and highest (20-fold) response to O_3 compared with the delayed induction (12 hours) of HSP70 and HO-1. HSP27 is expressed predominantly in the suprabasal epidermis in human skin, whereas HSP70 predominates in the dermis compared with the epidermis.

These differences in location between HSP27 and HSP70 might explain the different time course

of induction of these stress proteins upon O₃ exposure. It is therefore possible that the generated bioactive compounds may be responsible for the induction of HSPs as was also shown after UV irradiation.

(3) Induction of Matrix Metalloproteinases (MMPs)

Among the multiple systems altered in the skin by environmental pollutants, MMPs are among the major targets. Indeed, O₃ exposure is able to affect their synthesis and/or activity with logical consequences on tissue remodeling and wound healing^{23,24}. Within the MMP family, MMP-2 and MMP-9 are the only members able to degrade type-IV collagen of the basal membranes²⁵. MMP-2 is involved in pathological processes such as photoageing and precancerous/cancerous skin lesions after UV exposure; moreover, MMP-2 is capable of cleaving other substrates, in addition to type-IV collagen, including other MMPs and therefore can (indirectly) control extracellular matrix degradation and remodelling.

MMP-9, like MMP-2, plays a role in human skin ageing²⁶ tumor development²⁷, as well as in other cutaneous lesions such as psoriasis and dermatitis^{28,29}. In a recent study, we were able to demonstrate that O₃ was able to affect MMP activity. Most likely the generation of bioactive molecules can be the cause of such activation. It has been also demonstrated that O₃ is able to induce NO production via the activation of iNOS in cutaneous tissues¹⁸. When produced in excess, NO, may combine with superoxide to form peroxynitrite (derived from other sources) that can activate MMPs especially MMP-9. Thus, the increase of oxidative stress after exposure, plus the interaction between O₂ and nitrogen active molecules might be the main mechanism that leads to the enhanced MMPs activities in skin tissues. This can be also a result from an imbalance between MMPs and their endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs)³⁰.

In fact, the activities of MMPs are regulated by TIMPs, which can be produced by a multitude of cell types present in the cutaneous tissue. While MMP activity is altered by the O₃, neither TIMP-1 nor TIMP-2 level expression is affected. The lack of changes in TIMP-1 and 2 levels, combined with the increased activity of MMPs suggest that O₃ can cause a net increase in matrix degradation. On the other hand, in a comparative study where normal skin has been exposed for two hours to environmentally realistic levels of ozone, only a moderate state of oxidative stress at level of the stratum corneum has been induced, without producing a visible clinical response³¹.

4. Skin Age-Related Responses to Ozone Exposure: Wound Healing

Wound healing is a critical process in the skin and it has known to be affected by oxidative stress and also to decline with increasing age³². Although the exact sequence of wound healing is complex, cutaneous wound healing begins with wounding induced signaling factor-based transformation of stationary keratinocytes into cells capable of both replication and migration. Upon transformation, these cells express a host of molecules that promote the invasion of the injured epithelial matrix and reepithelialisation of the wound surface³³. Delayed wound healing in the elderly has been well described³⁴.

As mentioned above, O₃ exposure is also associated with activation of transcription factor NFκB, which is important to regulate inflammatory responses and eventually entire wound healing. O₃ exposure increased levels of Transforming Growth Factor (TGFβ) that is a critical factor in tissue remodeling^{35,36}. We can summarize that while O₃ as an oxidant, might stimulate wound healing, it would be detrimental in an "aging environment" due to the increased concentration-dependent oxidative stress. Therefore, these aspects have biological as well as practical implications and needed further investigations. In a recent study, we demonstrated the detrimental effects of O₃ on cutaneous wound healing in the aged animals. In fact, when hairless young (8-week-old) and aged mice (18-months-old) with after full thickness excisional wounds were exposed to 05 μg/mL O₃ for 6 hours per day the rate of wound closure was significantly delayed in the old group. We also showed induction of protein and lipid oxidation assessed as changes in protein oxidation (carbonyls) and lipid peroxidation (4-hydroxynonenal, HNE adducts) in the old mice compared to the young mice during the later stage of cutaneous wound healing. O₃ exposure has different effects depending on the age of the mice. In fact, it significantly delayed wound closure in old mice, while in young mice, it led to accelerated trend during the first few days of the exposure. This might be attributed to the antibacterial properties of O₃, as it has been shown that application of "hydropressive" ozonation provides fast cleansing of wound surface from pyonecrotic masses, promotes elimination of infection and thus substantially reduces the period of treatment of the patients³⁷. Recently, clinical treatments using hyperbaric oxygen therapy demonstrated that increased O₂ tension at the wound site increases the formation of granulation tissue, enhances accelerated wound closure and ameliorates impaired dermal wound healing³⁸; therefore, accelerated trend of wound closure shown in young

population may be due to decreased bacterial infection and/or increased O_2 tension by O_3 exposure in wound area.

One of the possible driving processes of the effect of O_3 on wound healing can be also in this case the modulation of the transcription factor NF κ B. Interestingly, the dose–effect relationship between level of oxidative stress and NF κ B exhibits a biphasic profile: while moderate levels of oxidative stress activate NF κ B through an I κ B kinase independent mechanism, extremely high levels of oxidative stress have been shown to inhibit NF κ B activation by blocking I κ B α phosphorylation³⁹. One potential explanation for the differential effect in the older animals is that the level of oxidative stress generated by O_3 exposure combined with aging causes levels of oxidative stress that inhibits I κ B α phosphorylation, thereby resulting in a decline in NF κ B activation. This finding is consistent with what mentioned previously that O_3 exposure induced skin antioxidants depletion.

This interpretation is also bolstered by data on TGF β a crucial modulator of tissue remodeling and is linked to both NF κ B status as well as to levels of oxidative stress during entire wound healing process⁴⁰. The reduced TGF β levels in both air and O_3 exposed old mice as well as the lower induction of TGF β by O_3 exposure in the old animals suggests that the noted delays in wound closure might be related to defects in oxidative stress-dependent NF κ B status as well as levels of oxidative stress and TGF β signaling in aged mice during later stage of wound healing.

5. Topical Application of Ozone in Medicine

To the best of our knowledge, the first application of gaseous O_3 was performed during World War I for treating German soldiers affected by gaseous gangrene due to *Clostridium anaerobic* infections very sensitive to O_3 ^{41,42}. In 1936, Dr P. Aubourg, by using a metal cannula, was the first to propose the insufflations of gaseous O_2/O_3 in the rectum to treat chronic colitis, anal ragadis and fistulae. This approach is very empirical and unprecise and today it is mostly used by Cuban physicians. In 1937, a Swiss dentist, E. A. Fisch (1899–1966) had the idea to use it in his practice and, by a twist of fate, he treated Dr. E. Payr (1871–1946) a surgeon who had a painful gangrenous pulpitis. Payr was so enthusiastic of the O_3 effect to use it in his surgical practice with great advantage⁴³. Later on, Werkmeister⁴⁴ mastered the use of gaseous O_3 in several skin ulcers due to atherosclerosis, diabetes and radiotherapy by either enclosing a leg in a polythene-bag (the so-called bagging system)

or using an ozone-resistant plastic cup applied in other areas. In the former application the gas was introduced to just inflate the bag containing some distilled water. The system was static but after 20–25 minutes the gas was aspirated and destroyed. The O_3 concentrations varied between a high 80 μ g/mL in very purulent ulcers and progressively lower concentrations down to 10 μ g/mL as the ulcers improved because excessive O_3 would be deleterious for healing. As the cup system had an inlet and an outlet, Werkmeister could realize a continuous gas flow with a modest depression that enhanced the vasodilation of the ulcer's area. With both systems he treated many extensive and otherwise incurable lesions within 50–200 days. It is noteworthy that gaseous O_3 works well only in a water vapour-saturated bag because it must dissolve into superficial water or in the exudate to react proficiently. The normal skin does not undergo any damage during the treatment. Today these procedures are still in use but they are somewhat cumbersome and great care must be exercised to prevent air contamination.

How ozonated oils act remains an open question. Probably, when the stable triozone comes into contact with the warm exudate of the wound, it slowly decomposes into different peroxides, which readily dissolves in water, probably generating hydrogen peroxide that can explain the prolonged disinfectant and stimulatory activity. If it is correct, this reasoning implies that we should have titrated preparations with high, medium, or low ozonide concentrations to be used during the inflammatory septic phase I, regenerating phase II or remodeling phase III, respectively². These phases have been related to the rapidly changing cell types and to the release of cytokines and growth factors that modulate the complex healing process.

An alternative method for treating diabetic foot ulcers is the use of hyperbaric oxygen therapy (HOT) but in such a case one disadvantage is the use of only hyperbaric O_2 and another is the need to close the patient in the chamber for two hours. Therapeutic results are far more modest than topical O_3 application, particularly when it is contained in a close cabinet with thermostatically-controlled temperature. However this procedure requires considerable idle times and, if an aspirating pump is unavailable, it may contaminate the operating room. For these reason today for cleaning and disinfecting cutaneous and mucosal infections and lesions due to many causes (like, e.g., trauma, ischemia, burns), it appears preferable to use at once freshly ozonated water and then ozonated oil, particularly during the night or at rest conditions.

The process of water ozonation needs of double distilled water and O_3 concentrations ranging from

20 up to 100 $\mu\text{g}/\text{mL}$ of gas to have a final yield of 5 up to 25 $\mu\text{g}/\text{mL}$, respectively. O_3 is directly bubbled into the water and the gas in excess is passed through a dehydrating device and finally through a destructor. Depending upon the water volume and the gas flow, a period of ozonation between 5–20 minutes is sufficient to saturate the water with gaseous O_3 . In fact, if the water is ultrapure, O_3 physically dissolves in the absence of chemical reactions and if kept in a glass bottle closed with a Teflon cap, the concentration halves only after 300 hours at 0°C . However, at 20°C the half-life is about 10 hours⁴⁵. It must be noted that monodistilled water allows a much faster O_3 decomposition and it is not practical. It is advised to maintain the bottle at 4°C and to quickly close the bottle at any time, or better to have a valve system to prevent gas losses. It would be useful to devise a procedure for maintaining the O_3 concentration for longer times and we are investigating a possible procedure. On the other hand, ozonation of either olive or sunflower oils requires a much longer time and the procedure needs to be well-standardized in terms of gas-flow, O_3 concentration, oil volume, and temperature. As recently reviewed, at least twenty different vegetable oils have been patented but so far it remains impossible to define their relative cost/benefit⁴⁶. At this stage, after evaluating several physicochemical criteria, stability, efficacy, and cost, it seems that sesame oil has several advantages in comparison to other oils.

How and when ozonated water and oils are used? Chronic wounds range from diabetic foot to putrid and deep ulcers due to limb atherosclerosis, or trauma and burns. Moreover, both immunosuppressive chemotherapy and/or malnutrition cause abscesses, anal fissures and fistulae, bed sores, furunculosis, and osteomyelitis which are difficult to treat and often fail after prolonged treatments. About 7 million patients in the United States are affected with a cost over US\$ 25 billion annually. Various types of disinfectants, antibiotics, antifungal, antiprotozoal, and growth factors are scarcely effective because the deranged metabolism and local hypoxia are not modified. Several other approaches such as vacuum therapy^{47,48}, maggot therapy⁴⁹ and devices for providing topical oxygen therapy in a clinical setting have been proposed and variably used. This last approach has a rationale in the sense that enhanced oxygenation is useful for activating the metabolism and cell proliferation of ischemic tissues⁵⁰⁻⁵². However, it has also considerable limitations because it is a cumbersome therapy, with minimal disinfectant activity and modifications of the fundamental pathogenetic mechanisms.

Another topic of critical interest is the pathologies of the vaginal mucosa. Although rarely deadly

(as the toxic shock syndrome due to a forgotten absorbent tampon), a majority of women physically and psychologically frequently suffer from a number of infections due to several pathogens such as *Neisseria gonorrhoeae*, *Trichomonas vaginalis*, *Candida albicans*, *Chlamidia trachomatis*, Herpes virus type-II (HV-II), human papilloma viruses (HPV), human immunodeficiency virus (HIV), often due to unprotected sexual intercourses, stress, change in sexual partners and also physiological hormonal changes during menopause. About 20 million Americans are affected by the distressing HV-II and as many 40 million have the genital HPV with warts and the impending risk of cervix cancer. Moreover, the further implantation of opportunistic infections complicates the treatment. It is unfortunate that orthodox medications are expensive and not so useful because of drug-resistant pathogens and side effects limiting the compliance. So far official medicine has not yet entertained the topical use of O_3 and derivatives in therapy because they are not profitable and no extensive clinical trials have been published in peer-reviewed journals: the therapy has remained in practitioners' hands and the results remain anecdotal. Moreover, the parenteral use of ozone, also known as ozone therapy, is very useful as adjuvant: it is reasonably easy to perform in terms of classical ozonated major and minor autohemotherapy⁵³. The latter modality has been successfully used for eliminating recurrences of HV-I and II infections. However, topical therapy is essential and it is carried out by using vaginal irrigation of fresh ozonated water and application of vaginal ozonated oil pessaries for the night. During prolonged treatment the ozonated compounds allows the elimination of any pathogens. So far no resistance to O_3 has been demonstrated. Creams containing ozonated oils can be used 3-4 times daily for external genital areas and also for several anorectal affections.

As for the oral infections (aphthae, HV-I, opportunistic superinfections, or acne) the earliest as possible application of ozonated ointments, by minimizing pathogen diffusion and enhancing microcirculation, reduces the swelling, destroys the pathogen, and allows a rapid healing.

Last but not least, clinical trials in tinea pedis as well as onychomycosis^{54,55} have been recently published and have shown the usefulness of ozonated sunflower oil.

6. Conclusions

At the present, especially in young people, venereal infections are increasingly frequent and therefore a suitable, effective medication with

ozonated compounds will be a huge economical and social value. Also, elderly people are burdened with a variety of wounds and ulcers, some of which never heal, making life miserable. It is hoped that

the present paper will inform official medicine for this advance and will incite to programme suitable clinical trials to show the full efficacy of ozone therapy by evidence-based medicine.

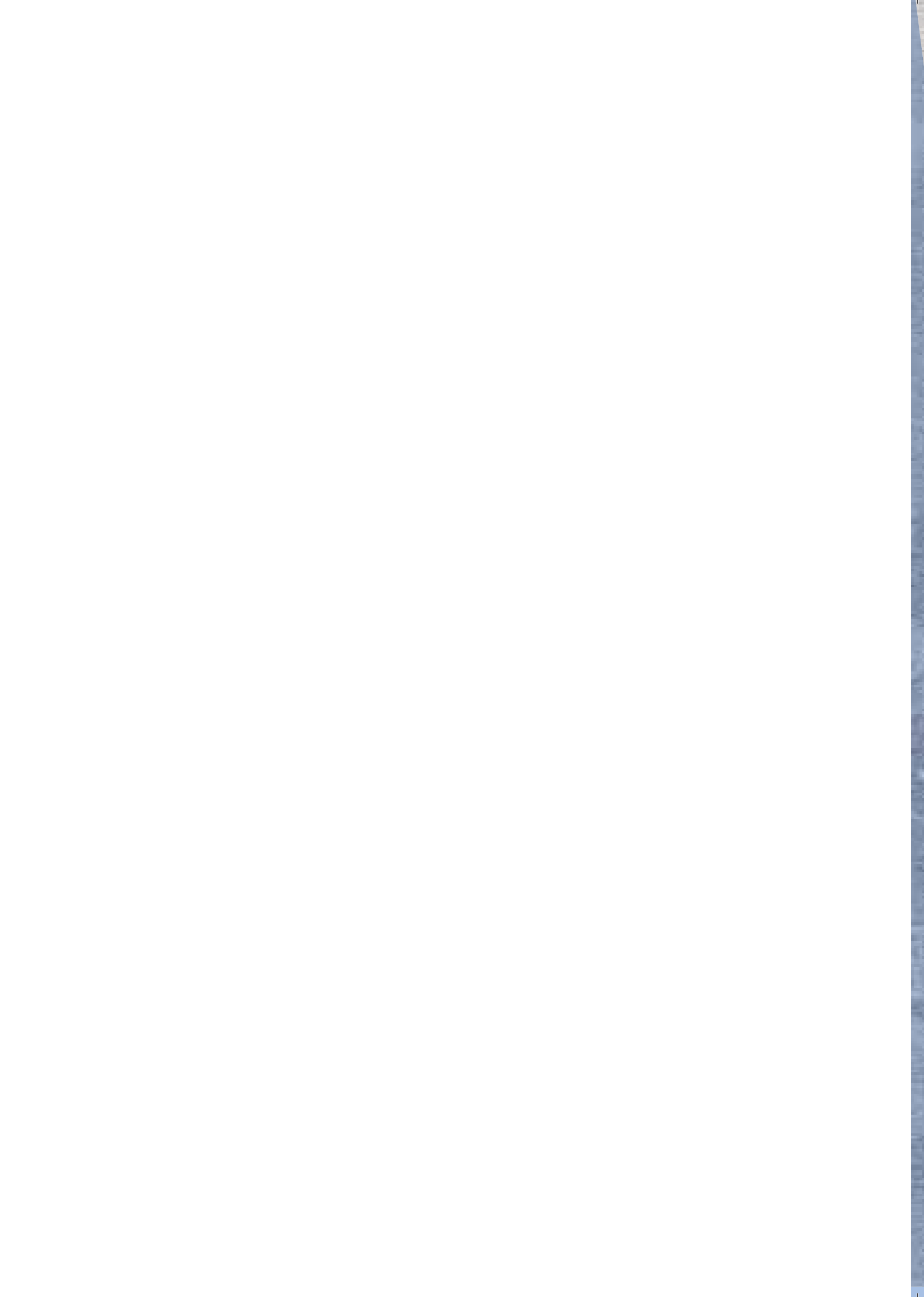
References

- 1 F. Werdin, M. Tenenhaus, and H.-O. Rennekampff, "Chronic wound care," *The Lancet*, vol. 372, no. 9653, pp. 1860-1862, 2008.
- 2 P. Martin, "Wound healing-aiming for perfect skin regeneration," *Science*, vol. 276, no. 5309, pp. 75-81, 1997.
- 3 N. Yamada, W. Li, and W. Li, "Platelet-derived endothelial cell growth factor gene therapy for limb ischemia," *Journal of Vascular Surgery*, vol. 44, no. 6, pp. 1322-1328, 2006.
- 4 N. Papanas and E. Maltezos, "Growth factors in the treatment of diabetic foot ulcers: new technologies, any promises?" *The International Journal of Lower Extremity Wounds*, vol. 6, no. 1, pp. 37-53, 2007.
- 5 H.S. Kim, S.U. Noh, Y.W. Han, K.M. Kim, H. Kang, H.O. Kim, and Y.M. Park, "Therapeutic effects of topical application of ozone on acute cutaneous wound healing," *Journal of Korean Medical Science*, vol. 24, no. 3, pp. 368-374, 2009.
- 6 G. Valacchi, Y. Lim, I. Zanardi, V. Bocci, and V. Travagli, "Evaluation of ozonated sesame oil effect in wound healing using the SKH1 mice as a model," in *Proceeding of the 7th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology*, Valletta, Malta, March 2010.
- 7 A. Napolitano, L. Panzella, M. Savarese, R. Sacchi, I. Giudicianni, L. Paolillo, and M. D'Ischia, "Acid-induced structural modifications of unsaturated fatty acids and phenolic olive oil constituents by nitrite ions: a chemical assessment," *Chemical Research in Toxicology*, vol. 17, no. 10, pp. 1329-1337, 2004.
- 8 R.T. Rappolt Sr., "The ozone generator," *Clinical Toxicology*, vol. 5, no. 3, pp. 419-425, 1972.
- 9 A. Sega, I. Zanardi, L. Chiasserini, A. Gabbrielli, V. Bocci, and V. Travagli, "Properties of sesame oil by detailed ¹H and ¹³C NMR assignments before and after ozonation and their correlation with iodine value, peroxide value, and viscosity measurements," *Chemistry and Physics of Lipids*, vol. 163, no. 2, pp. 148-156, 2010.
- 10 I. Zanardi, V. Travagli, A. Gabbrielli, L. Chiasserini, and V. Bocci, "Physico-chemical characterization of sesame oil derivatives," *Lipids*, vol. 43, no. 9, pp. 877-886, 2008.
- 11 G. Martinez Tellez, O. Ledea Lozano, and M. Díaz Gómez, "Measurement of peroxidic species in ozonized sunflower oil," *Ozone: Science and Engineering*, vol. 28, no. 3, pp. 181-185, 2006.
- 12 D.J.P. Bassett, E. Bowen-Kelly, C.L. Elbon, and S.S. Reichenbaugh, "Rat lung recovery from 3 days of continuous exposure to 0.75 ppm ozone," *Journal of Toxicology and Environmental Health*, vol. 25, no. 3, pp. 329-347, 1988.
- 13 L. Fiévez, N. Kirschvink, and N. Kirschvink, "Impaired accumulation of granulocytes in the lung during ozone adaptation," *Free Radical Biology and Medicine*, vol. 31, no. 5, pp. 633-641, 2001.
- 14 O.R. Moss, E.A. Gross, and E.A. Gross, "Respiratory tract toxicity in rats exposed to Mexico City air," *Research Report 100*, Health Effects Institute, 2001.
- 15 L. van Bree, J.A.M.A. Dormans, A.J.F. Boere, and P.J.A. Rombout, "Time study on development and repair of lung injury following ozone exposure in rats," *Inhalation Toxicology*, vol. 13, no. 8, pp. 703-718, 2001.
- 16 J.J. Thiele, M.G. Traber, R. Re, N. Espuno, L.-J. Yan, C.E. Cross, and L. Packer, "Macromolecular carbonyls in human stratum corneum: a biomarker for environmental oxidant exposure?" *FEBS Letters*, vol. 422, no. 3, pp. 403-406, 1998.
- 17 G. Valacchi, A. van der Vliet, B.C. Schock, T. Okamoto, U. Obermuller-Jevic, C.E. Cross, and L. Packer, "Ozone exposure activates oxidative stress responses in murine skin," *Toxicology*, vol. 179, no. 1-2, pp. 163-170, 2002.
- 18 G. Valacchi, E. Pagnin, and E. Pagnin, "Induction of stress proteins and MMP-9 by 0.8 ppm of ozone in murine skin," *Biochemical and Biophysical Research Communications*, vol. 305, no. 3, pp. 741-746, 2003.
- 19 G. Valacchi, E. Pagnin, A.M. Corbacho, E. Olano, P.A. Davis, L. Packer, and C.E. Cross, "In vivo ozone exposure induces antioxidant/stress-related responses in murine lung and skin," *Free Radical Biology and Medicine*, vol. 36, no. 5, pp. 673-681, 2004.
- 20 G. Valacchi, A. Pecorelli, M. Mencarelli, E. Maioli, and P.A. Davis, "Beta-carotene prevents ozone-induced proinflammatory markers in murine skin," *Toxicology and Industrial Health*, vol. 25, no. 4-5, pp. 241-247, 2009.
- 21 N.Y. Schurer, G. Plewig, and P.M. Elias, "Stratum corneum lipid function," *Dermatologica*, vol. 183, no. 2, pp. 77-94, 1991.
- 22 N.Y. Schürer and P.M. Elias, "The biochemistry and function of stratum corneum lipids," *Advances in Lipid Research*, vol. 24, pp. 27-56, 1991.
- 23 S.U. Weber, J.J. Thiele, C.E. Cross, and L. Packer, "Vitamin C, uric acid, and glutathione gradients in murine stratum corneum and their susceptibility to ozone exposure," *Journal of Investigative Dermatology*, vol. 113, no. 6, pp. 1128-1132, 1999.
- 24 P. Brenneisen, H. Sies, and K. Scharffetter-Kochanek, "Ultraviolet-B irradiation and matrix metalloproteinases: from induction via signaling to initial events," *Annals of the New York Academy of Sciences*, vol. 973, pp. 31-43, 2002.
- 25 L.J. McCawley and L.M. Matrisian, "Matrix metalloproteinases: they're not just for matrix anymore!," *Current Opinion in Cell Biology*, vol. 13, no. 5, pp. 534-540, 2001.
- 26 V. Koivukangas, M. Kallioinen, H. Autio-Harmanen, and A. Oikarinen, "UV irradiation induces the expression of gelatinases in human skin in vivo," *Acta Dermatovenereologica*, vol. 74, no. 4, pp. 279-282, 1994.
- 27 F. Rijken, R.C. M. Kiekens, and P.L. B. Bruijnzeel, "Skin-infiltrating neutrophils following exposure to solar-simulated radiation could play an important role in photoageing of human skin," *British Journal of Dermatology*, vol. 152, no. 2, pp. 321-328, 2005.
- 28 S. Suomela, A.-L. Kariniemi, E. Snellman, and U. Saarialho-Kere, "Metalloelastase (MMP-12) and 92-kDa gelatinase (MMP-9) as well as their inhibitors, TIMP-1 and -3, are expressed in psoriatic lesions," *Experimental Dermatology*, vol. 10, no. 3, pp. 175-183, 2001.
- 29 A.C.A. Devillers, A.W. van Toorenbergen, G.J. Klein Heerenbrink, P.G.H. Mulder, and A.P. Oranje, "Elevated levels of plasma matrix metalloproteinase-9 in patients with atopic dermatitis: a pilot study," *Clinical and Experimental Dermatology*, vol. 32, no. 3, pp. 311-313, 2007.
- 30 J. Zhang, Y.-J. Cao, Y.-G. Zhao, Q.-X. A. Sang, and E.-K. Duan, "Expression of matrix metalloproteinase-26 and tissue inhibitor of metalloproteinase-4 in human normal cytrophoblast cells and a choriocarcinoma cell line, JEG-3," *Molecular Human Reproduction*, vol. 8, no. 7, pp. 659-666, 2002.

- 31 Q.C. He, A. Tavakkol, K. Wietecha, R. Begum-Gafur, S.A. Ansari, and T. Polefka, "Effects of environmentally realistic levels of ozone on stratum corneum function," *International Journal of Cosmetic Science*, vol. 28, no. 5, pp. 349-357, 2006.
- 32 C.B. Ballas and J.M. Davidson, "Delayed wound healing in aged rats is associated with increased collagen gel remodeling and contraction by skin fibroblasts, not with differences in apoptotic or myofibroblast cell populations," *Wound Repair and Regeneration*, vol. 9, no. 3, pp. 223-237, 2001.
- 33 S. Werner and R. Grose, "Regulation of wound healing by growth factors and cytokines," *Physiological Reviews*, vol. 83, no. 3, pp. 835-870, 2003.
- 34 G.L. Grove and A.M. Kligman, "Age-associated changes in human epidermal cell renewal," *Journals of Gerontology*, vol. 38, no. 2, pp. 137-142, 1983.
- 35 G. Valacchi and V. Bocci, "Studies on the biological effects of ozone: 10. Release of factors from ozonated human platelets," *Mediators of Inflammation*, vol. 8, no. 4-5, pp. 205-209, 1999.
- 36 A. Leask and D.J. Abraham, "The role of connective tissue growth factor, a multifunctional matricellular protein, in fibroblast biology," *Biochemistry and Cell Biology*, vol. 81, no. 6, pp. 355-363, 2003.
- 37 V.I. Bulynin, A.I. Ermakova, A.A. Glukhov, and I.P. Mozhuróv, "Wound treatment using the flow of an ozonized solution under high pressure," *Khirurgiia*, no. 8, pp. 23-24, 1998.
- 38 P.K. Gajendrareddy, C.K. Sen, M.P. Horan, and P.T. Marucha, "Hyperbaric oxygen therapy ameliorates stress-impaired dermal wound healing," *Brain, Behavior, and Immunity*, vol. 19, no. 3, pp. 217-222, 2005.
- 39 M.-S. Byun, K.-I. Jeon, J.-W. Choi, J.-Y. Shim, and D.-M. Jue, "Dual effect of oxidative stress on NF- κ B activation in HeLa cells," *Experimental and Molecular Medicine*, vol. 34, no. 5, pp. 332-339, 2002.
- 40 A. Leask and D. J. Abraham, "TGF- β signaling and the fibrotic response," *The FASEB Journal*, vol. 18, no. 7, pp. 816-827, 2004.
- 41 G. Stoker, "The surgical use of ozone," *The Lancet*, vol. 188, no. 4860, p. 712, 1916.
- 42 G. Stoker, "The surgical use of ozone," *The Lancet*, vol. 189, no. 4891, p. 797, 1917.
- 43 E. Hanhart, "Über Ozonbehandlung in der Chirurgie," *Munchener medizinische Wochenschrift*, vol. 82, pp. 220-291, 1935.
- 44 H. Werkmeister, "Dekubitalgeschwüre und die Behandlung mit der Ozon-Unterdruckbegasung," in *Ozon-Handbuch. Grundlagen. Prävention. Therapie, V-7.1*, R. Viebahn-Hänsler, H. G. Knoch, et al., Eds., pp. 1-22, 2001.
- 45 R. Viebahn-Hänsler, *The Use of Ozone in Medicine*, ODREI Publishers, 5th edition, 2007.
- 46 V. Travagli, I. Zanardi, and V. Bocci, "Topical applications of ozone and ozonated oils as anti-infective agents: an insight into the patent claims," *Recent Patents on Anti-Infective Drug Discovery*, vol. 4, no. 2, pp. 130-142, 2009.
- 47 S.H. Wu, P.J. Zecha, R. Feitz, and S.E.R. Hovius, "Vacuum therapy as an intermediate phase in wound closure: a clinical experience," *European Journal of Plastic Surgery*, vol. 23, no. 4, pp. 174-177, 2000.
- 48 M. van den Boogaard, E. de Laat, P. Spauwen, and L. Schoonhoven, "The effectiveness of topical negative pressure in the treatment of pressure ulcers: a literature review," *European Journal of Plastic Surgery*, vol. 31, no. 1, pp. 1-7, 2008.
- 49 S. Hunter, D. Langemo, P. Thompson, D. Hanson, and J. Anderson, "Maggot therapy for wound management," *Advances in Skin & Wound Care*, vol. 22, no. 1, pp. 25-27, 2009.
- 50 G.M. Gordillo, S. Roy, S. Khanna, R. Schlanger, S. Khandelwal, G. Phillips, and C. K. Sen, "Topical oxygen therapy induces vascular endothelial growth factor expression and improves closure of clinically presented chronic wounds," *Clinical and Experimental Pharmacology and Physiology*, vol. 35, no. 8, pp. 957-964, 2008.
- 51 G.M. Gordillo and C.K. Sen, "Evidence-based recommendations for the use of topical oxygen therapy in the treatment of lower extremity wounds," *The International Journal of Lower Extremity Wounds*, vol. 8, no. 2, pp. 105-111, 2009.
- 52 C.K. Sen, G.M. Gordillo, and G.M. Gordillo, "Human skin wounds: a major and snowballing threat to public health and the economy," *Wound Repair and Regeneration*, vol. 17, no. 6, pp. 763-771, 2009.
- 53 V. Bocci, E. Borrelli, V. Travagli, and I. Zanardi, "The ozone paradox: ozone is a strong oxidant as well as a medical drug," *Medicinal Research Reviews*, vol. 29, no. 4, pp. 646-682, 2009.
- 54 S. Menéndez, L. Re, and L. Re, "Safety of topical Oleozon® in the treatment of tinea pedis: phase IV clinical trial," *International Journal of Ozone Therapy*, vol. 7, no. 1, pp. 55-59, 2008.
- 55 S. Menéndez, L. Falcón, and Y. Maqueira, "Therapeutic efficacy of topically Oleozon® in patients suffering from onychomycosis," *Mycoses*. In press.

Copyright © 2010 Hindawi Publishing

V. Travagli, MD
 Dipartimento Farmaco Chimico Tecnologico
 Università degli Studi di Siena
 Viale Aldo Moro 2
 53100 Siena, Italy





COMUNE DI BRESCIA



Federazione Italiana di
OSSIGENO-OZONOTERAPIA



Word Federation Oxygen
Ozone Therapy

SECOND ANNOUNCEMENT

III WORLD CONGRESS OF Oxygen-Ozone Therapy

V CONGRESSO NAZIONALE F.I.O.

Museo della Mille Miglia
from 14th to 16th April 2011
Brescia Italy

SCIENTIFIC SECRETARIAT

Chairman: Prof. Matteo Bonetti

ORGANIZING SECRETARIAT

Koinè Eventi snc
Via Fontane, 24
25133 Brescia
Tel 030.2002844 - Fax 030.2096783
info@koineeventi.com
www.koineeventi.com

Dear Colleagues,

The 3rd World Congress of the Ozone Therapy Federation which follows those held in Beijing and Madrid wants to offer a further scientific value in a sector where approximation and vague knowledge lead - let me say - to a mere justification of a much easier indifference.

This is not acceptable and this congress is another important meeting occasion to give validating certainties to physicians operating in this discipline. Recent clinical results of national and international case history show a need for a bigger effort in the research of rationality in the way this matter is dealt with. This 3rd World Congress then gives itself ambitious targets. It wants to fight deception and re-establish incontestable truth which is only possible with concrete knowledge. It wants to give credit to all those around the world who have been able to give certainty to this therapy. It wants to give the opportunity of a correct approach and give an occasion of deep analysis.

For all this I feel honoured to organize the WFOOT 3rd World Congress of Ozone Therapy for the first time in Italy. A big opportunity for all those who want to collaborate, compare, give the results of their own knowledge and experience, but above all for those who are humble enough to ask themselves "why?"

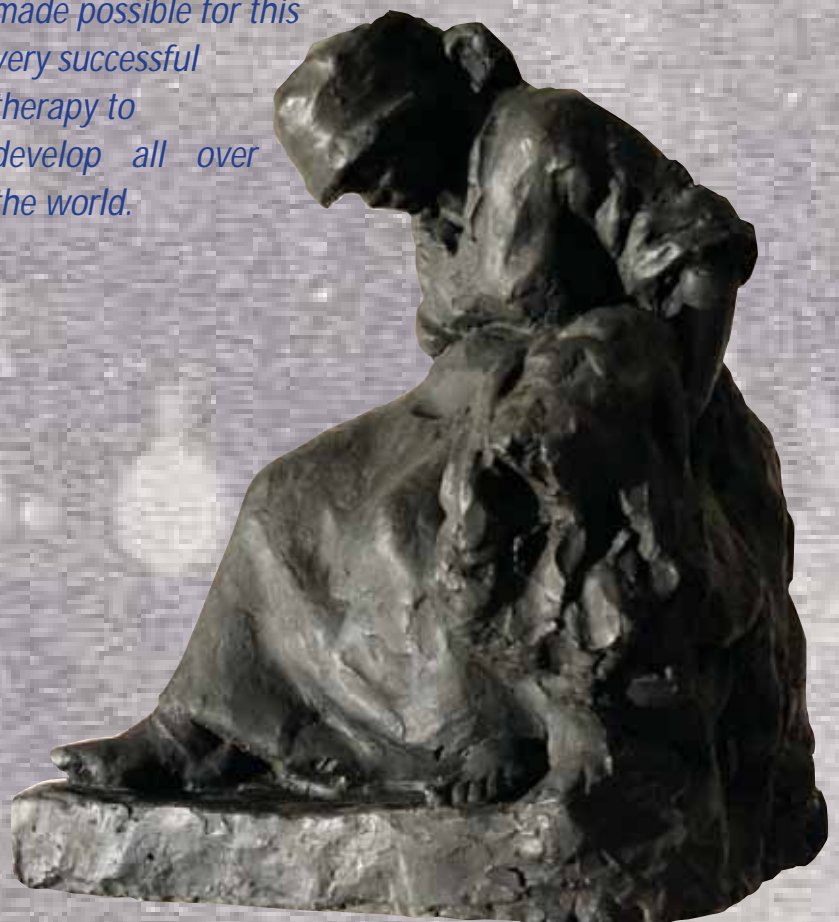
Thank you all

Matteo Bonetti

The Francesco Riccardo Monti prize for life time achievements is a recognition for scientific work done to spread Oxygen-Ozone Therapy practice in Italy and around the world.

The great artist Francesco Riccardo Monti, who is now represented by his heirs, was a sculptor and an architect from Cremona and he created many monumental works of art. At the end of 1928 after winning a commission and being prevented from completing it he had an argument with a fascist party official and left Italy. He moved first to France and then to Manila in the Philippines where within a short time he became the most important sculptor and architect in the country. There he carried out great works of art merging the style of the European school with the local tradition. He came back to Italy only for short periods between 1930 and 1932 to finish some uncompleted works.

His style is full of poetic symbolism and leads to works of art rich in grace and imagination. His devotion is the same of those physicians who have always believed in the effectiveness of Oxygen-Ozone Therapy and have made possible for this very successful therapy to develop all over the world.



Organizer Committee

Albertini Filippo - Italy
Alexandre Alberto - Italy
Borrelli Emma - Italy
Cardelli Roberto - Italy
D'Orta Giovanni - Italy
Dall'Aglio Roberto - Italy
De Santis Fabio - Italy
Fabris Giuliano - Italy
Fontana Alessandro - Italy
Genovese Eugenio - Italy
Gjonovich Antonio - Italy
Lamberto Re - Italy
Morosi Claudio - Italy
Muto Mario - Italy
Ottaviani Gian Maria - Italy
Parodi Filippo - Italy
Sirito Mario - Italy
Tabaracci Gabriele - Italy
Zambello Alessio - Italy

International Scientific Committee

Alvarado Ramiro - Bolivia
Andreula Cosma - Italy
Baeza Noci José - Spain
Bas Murat - Turkey
Bergeron Yves - Canada
Bocci Velio - Italy
Catelani Cardoso Cláudia - Brazil
Clavo Bernardino - Spain
Erario Angeles - Argentina
Gallucci Massimo - Italy
Galvan Francisco J. - Spain
Gomez Manuel - Cuba
He Xiaofeng - China
Hernández Frank - Cuba
Konrad Heinz - Brazil
Kos Wally - Australia
Kumar Vijay Sheel - India
Leonardi Marco - Italy
Mawsouf Nabil - Egypt
Menéndez Silvia - Cuba
Pellicanò Giannantonio - Italy
Pepa Osvaldo Alberto - Argentina
Sarchilli Alberto - Italy
Schulz Siegfried - Germany
Schwartz Tapia Adriana - Spain
Ungureanu Florin Dan - Romania
Viti Paganelli Sergio - Venezuela
Vyletelka Juray - Slovakia

Thursday 14 april

- 15.30-16.00 Registration of participants
16.00-16.30 Opening Ceremony
Welcome of local Authorities
16.30-17.30 **V. Kumar**
Ozone Therapy Today
17.30-18.00 **Francesco Riccardo Monti**
Awards Ceremony
18.00-19.00 **G. Pellicanò**
History of Rock
19.00 - 19.30 Visit of "Mille Miglia" Museum

Friday 15

I Session - Ozone Therapy

- 08.30-09.15 **V. Bocci**
State of the Art
09.15-09.35 **Fahmy**
New Frontiers of Pain Therapy

Oral Communications

II Session - Ozone Therapy in the Musculo-Skeletal Pathology

PART I - Spine

Moderators: **C. Andreula, O.A. Pepa**

- 09.35-09.55 **M. Leonardi**
State of the Art
09.55-10.15 **Y. Bergeron**
The Future of Minimally Invasive Treatment of the Spine: Role Of Ozone Therapy
10.15-10.35 **J. Baeza Noci**
Therapeutical Approaches
10.35-10.50 **Coffee Break**
10.50-11.10 **M. Gallucci**
Other Ozone Therapeutical Approaches: Facet Joints and Posterior Arch
11.10-11.30 **G. Pellicanò**
Review of International Literature
11.30-11.50 **Discussion**

Oral Communications

PART II - Articular

Moderators: **M. Bas, H. Konrad**

- 11.50-12.10 **X. He**
The Research on Degenerative Knee Cartilage in Rats
12.10- 12.30 **M. Muto**
The Research: Modifications Induced by Ozone In the Disk
12.30-12.50 **E. Genovese**
Mininvasives Treatments of Small and Big Articulations Compared to Ozone
12.50-13.10 **A. Scarchilli**
The Experience in Shoulder and Knee Treatment

Oral Communications

- 13.10 - 14.30 Lunch

III Session - Randomized Clinical Studies

Moderators: **F.D. Ungureanu, J. Vyletelka**

- 14.30-14.50 **M. Gallucci**
Ozone Versus Steroid in the Treatment of Lumbar Pain
14.50-15.10 **A. Zambello**
Epidural Infiltrations Versus Ozone Treatment
15.10-15.30 **F.J. Galvan**
Ozone Therapy Versus Microsurgery in the Treatment of Slipped Disk
15.30- 15.50 **L. Re**
Clinical Evaluation of the Pain in the Elderly Patient, Ozone Versus Traditional Pharmacological Treatment

Oral Communications

- 15.50-16.10 **Coffee Break**

IV Session - World Experiences

Moderators: **J. Baeza Noci, A. Alexandre**

- 16.10-16.30 **W. Kos** - Oceania
16.30-16.50 **V.S. Kumar** - Asia
16.50-17.10 **C. Andreula** - Europe
17.10-17.30 **R. Alvarado** - America
17.30-17.50 **Nabil Massouf** - Africa

Other Applications

Moderators: **F. Hernandez-L.Re**

- 17.50-18.10 **S. Schulz**
Tumours
18.10-18.30 **A. Schwarz Tapia**
Ozone Therapy in Recurrent Vulvovaginal Candida Albicans Infections
18.30-18.50 **S. Menéndez**
Diabetes

Oral Communications

Saturday 16

Session - Veterinary Medicine

Moderators: **G. Penocchio, A. Corradi**

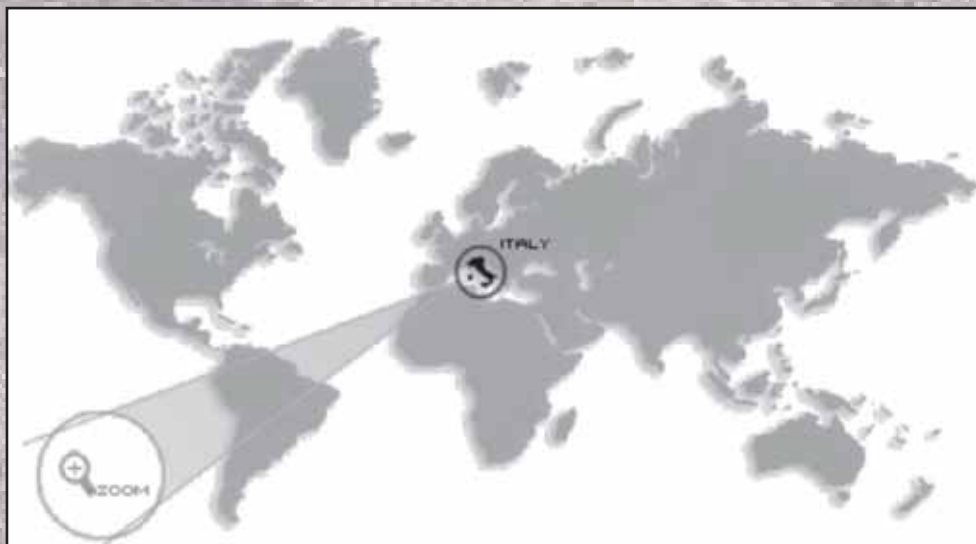
- 09.00-09.30 **H. Giuliano** - (Small Animals)
09.30 -10.00 **P. Scrollavezza**
Cow Mastitis Treatment
10.00-10.30 **E. Ballardini**
The Big Hemotransfusion in Horses
10.30-11.00 **E. Smadelli**
Practical Applications and Clinical Cases (Small Animals)
11.00-11.20 **Coffee Break**
11.20-12.20 **C. Dimauro**
Practical Applications and Clinical Cases (Small Animals)
12.20-13.00 **M. Ablondi**
Practical Applications and Clinical Cases (Cows)

Oral Communications

- 13.00-13.30 **Discussion of Posters**

End of works at 13:00 p.m.

HOW TO REACH THE MUSEUM



LOCATION OF THE MUSEUM

The Museum is situated inside the Monastery of Saint Eufemia, founded in years 1008. Saint Eufemia is a district lying on the east side of Brescia, on the 45Bis "Gardesana Occidentale" Main Road: Viale della Rimembranza that is between Via Indipendenza and Via della Parrocchia.

GPS NAVIGATOR:

TomTom Navigator and similar:

Lat= 45.52411 N - Lon= 10.26783 E - Garmin, eTrex e simili:

Lat= 45° 31'.446 N - Lon= 10° 16'.0698 E

How to reach us:

By car:

A4 and A21 Highway "Brescia Centro" exit

Follow direction to S. Eufemia, Proceed along Via Maggio, Via Mensi, Via Fiorentini, Via Gatti, Via Zammarchi, Viale S. Eufemia.

A4 Highway: "Brescia Est" exit

Follow direction to Brescia along the South ring road S. Eufemia exit. Follow direction to S. Eufemia. Proceed along Via Serenissima, Viale Sant'Eufemia

From the city centre of Brescia:

Piazzale Arnaldo, Viale Venezia della Mille Miglia, Viale della Bornata, S. Eufemia

By bus: From the city: n. 3 Urban line (Rezzato direction) and n. 11 from Garda Lake bus stop "Eufemia" Others extra urban lines: station bus stop of Brescia and then urban lines.

By train: Brescia Station, Bus line n. 3 (Rezzato direction) and n. 11, Taxi

By plane: G. D'Annunzio di Montichiari Airport, Shuttle bus from/to Bus Station, Line bus n. 3 (Rezzato direction) and n. 11

GENERAL INFORMATION

CONFERENCE DATE AND LOCATION

The conference will take place in S. Eufemia (BS) at the Mille Miglia Museum Viale della Rimembranza, 3 on 14th, 15th and 16th April 2011.

ABSTRACTS

Submitted abstracts related to the topics of the conference will be selected for presentation as poster or oral communications.

Abstracts will be published on the International Journal Of Ozone Therapy.

All abstracts must be in English and must mention authors and their affiliations. They must contain title, goals, materials and methods, results and conclusions.

Maximum length allowed for each abstract is 3000 characters including spaces.

Please send your abstracts to info@koineeventi.com

Deadline for abstracts submission is February 1st 2011

REGISTRATION FEE

	Before February 1st	After February 1st
Regular fee:	<input type="checkbox"/> 250 €	<input type="checkbox"/> 350 €
F.I.O. Members:	<input type="checkbox"/> 100 €	<input type="checkbox"/> 200 €
W.F.O.O.T Members:	<input type="checkbox"/> 100 €	<input type="checkbox"/> 200 €

Registration fee includes:

congress kit
coffee break
lunch

HOW TO PAY FOR REGISTRATIONS

It is possible to pay by cheque or bank transfer in favour of Koinè eventi (see attached registration form). Koinè eventi will send the invoice to the participant or to the paying company/institution.

Note: in case of reservation paid by public or private institution you are kindly requested to provide us with an authorization written on the institution letterhead with all invoicing details (name, address, VAT No.).

Please send the authorization along with the reservation form

HOTEL RESERVATIONS

(Prices are available in the reservation form).

Koinè eventi has reserved a certain number of rooms at the Villa Fenaroli Palace Hotel especially for the conference. To make a reservation please fill in the attached form and send it to Koinè eventi to allow them to reserve the room/rooms depending on availability and according to your request. After the reservation has been made successfully Koinè will send you a voucher with the full address of the hotel.

English will be the official language of the conference

III WORLD CONGRESS OF OXYGEN – OZONE THERAPY

V CONGRESSO NAZIONALE F.I.O.

Title _____ Name _____ Surname _____

Date of birth _____ Place of birth _____

Home address _____

Zip code _____ Town _____ Province _____ Country _____

Phone _____ Fax _____ e-mail _____

WORK ADDRESS:

Service/Division/Institute _____

Company/University _____

Address _____

Zip code _____ Town _____ Province _____ Country _____

Phone _____ Fax _____ e-mail _____

PAYMENT:

	Before February 1st	After February 1st
Regular fee:	<input type="checkbox"/> 250 €	<input type="checkbox"/> 350 €
F.I.O. Members:	<input type="checkbox"/> 100 €	<input type="checkbox"/> 200 €
W.F.O.O.T Members:	<input type="checkbox"/> 100 €	<input type="checkbox"/> 200 €

THE PAYMENT HAS BEEN MADE BY:

Bank cheque number _____

of Bank _____ in favour of Koinè eventi snc

The cheque must be sent, possibly by registered mail, to the organizing Secretariat Koinè eventi snc
Via Fontane, 24 - 25133 Brescia, together with the registration form.

Bank to bank transfer in favour of Koinè eventi snc

UBI - Banco di Brescia F.le n. 27 - Via Ambaraga n. 126 (BS) c/c 19000 - CIN: R- ABI 3500 - CAB 11290
IBAN: IT40R0350011290000000019000 BIC/SWIFT : BCAIT21090

INVOICE DATA:

Name _____

Address _____

Fiscal Code (Italian) _____

VAT _____

Please return to: **Koinè eventi snc**, via fontane, 24 - 25133 Brescia - Italy

Fax: +39 030.2096783 - e-mail: info@koineeventi.com

III WORLD CONGRESS OF OXYGEN – OZONE THERAPY V CONGRESSO NAZIONALE F.I.O.

HOTEL RESERVATION FORM

Name _____

Surname _____

Home Address _____

ZIP _____ City _____ Province _____ Country _____

Telephone _____ Fax _____

E-mail _____ Mobile _____

I would like to make a reservation as follows:

Room: n. 1 single double use n. 1 double

Arrival date _____ **Departure date** _____ **No. Of nights** _____

Double single use € 84,00

Double € 104,00

To guarantee my reservation I agree to give my credit card details to Koinè eventi who will forward them to the hotel.

Card type:

VISA **MASTERCARD** **AMERICAN EXPRESS** **DINERS**

Card n. _____ Card holder _____

Expiry date _____ Card holder signature _____

The agency will reserve the room/rooms depending on availability and according to participant's requirement. Reservation forms must be sent to Koinè eventi within March 15th. Requests of reservation sent after that date can't be guaranteed. Each request will be forwarded to the hotel.

The contract is made between the customer and the hotel. Koinè will send a voucher showing all details about the reservation.

Payment will be made directly to the hotel that will issue the related invoice at departure.

Approved by (card holder signature): _____

Note: in case of reservation paid by public or private institution you are kindly requested to provide us with an authorization written on the institution letterhead with all invoicing details (name, address, VAT No.).

Please send the authorization along with the reservation form.

Date _____ Signature _____

Please return to: **Koinè eventi snc**, via fontane, 24 - 25133 Brescia - Italy

Fax: +39 030.2096783 - e-mail: info@koineeventi.com

Errata-Corrige

Non-Invasive Approaches to Back Pain in Patients with Somatization

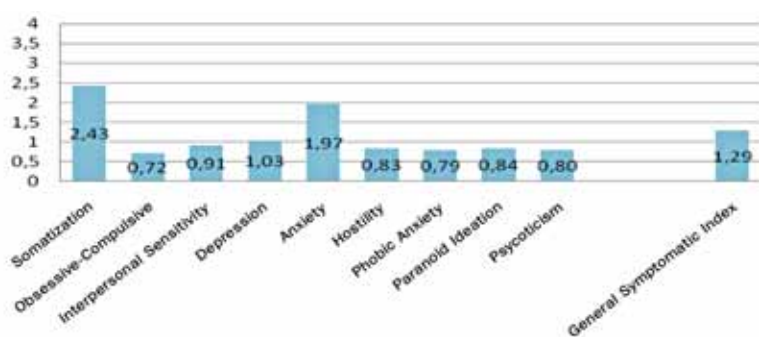
A. BARISELLI

Clinical Psychologist, Intensive Short Term Dynamic Psychotherapy

Key words: back pain, psychosomatic, somatization, I.S.T.D.P., S.C.L.90

Results

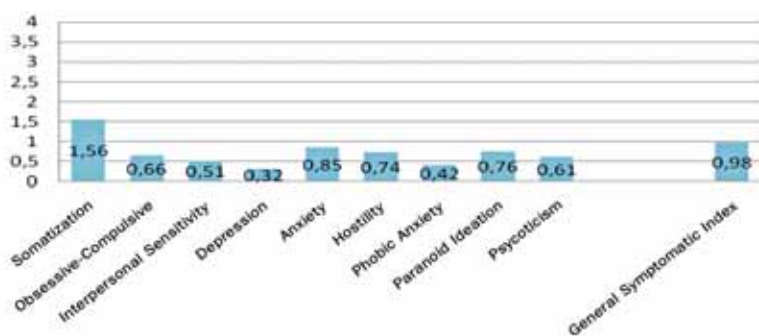
The first test administration yielded the following results:



Additional scales:

General neuroticism	1.11
Sleep disorders	3.33
Distress	1.41
Difficulty in cognitive performance	1.38

In the second administration we obtained these indexes:



Additional scales:

General neuroticism	0.82
Sleep disorders	1.23
Distress	0.81
Difficulty in cognitive performance	0.64

Spine Radiology

Diagnostic & Interventional
2nd Joint Meeting of ASSR and ESNR

Barcelona, Spain, 1-3 July 2011

Hotel NH Costanza

Program Committee:

Walter Bartynski, Bassem Georgy, Mario Muto,
Greg Petermann, Majda Thurnher, Johan Van Goethem

Local Organizer:

Alex Rovira

Topics:

Advanced Imaging Techniques, Pediatric Spine, Degenerative
Disease, Tumors, Infection and Inflammation,
Vertebroplasty and Kyphoplasty, Spine Interventions,
Spinal Trauma, Pain Management

www.spineinternational.org





World Federation Oxygen - Ozone Therapy

World Federation of Oxygen - Ozone Therapy

PRESIDENT

Dr. Vijam Sheel Kumar
Kumar Pain Management
& Speciality Centre
D.1/28 Vasant Vihar
110057 New Delhi
Tel.: 91 11 26142282 - 26154106
mail: spineconf@kpmc.com

VICE-PRESIDENT

Dr. Cosma Andreula
Anthea Hospital
Via C. Rosalba, 35-37
70124 Bari (BA)
Tel.: 080/550411
Fax: 080/5042487
mail: cosmaandreula@tin.it

SECRETARY GENERAL

Dr. Matteo Bonetti
Istituto Clinico Città di Brescia
Via Gualla, 15
25123 Brescia (BS)
Tel.: 030/3710311
Fax: 030/3710370
mail: matbon@numerica.it

TREASURER

Dr. Alessandro Fontana,
Istituto Clinico Città di Brescia
Via Gualla, 15
25123 Brescia (BS)
Tel.: 030/3710311
Fax: 030/3710370
mail: saratas@tin.it

CENTRAL OFFICE

X Ray Service s.r.l.
Via Oberdan, 140
25128 Brescia (BS)
Tel.: 030/3701312
Fax: 030/3387728
mail: info@xrayservice.it

Membership fees may be paid as follows:

by bank transfer free of charge credited to the F.I.O. - W.F.O.T.
account

BANCA CARIGE - Agenzia 2 - BRESCIA - ITALIA - € 125,00

IBAN: IT 35 K061 7511 2020 0000 0624 780 - BIC: CRGEITGG542

Please do not forget to indicate your name on the bank transfer.

Sincerely yours,

Dr. A. Fontana

W.F.O.O.T. (World Federation of Oxygen - Ozone Therapy)



World Federation Oxygen - Ozone Therapy

World Federation of Oxygen - Ozone Therapy

First name Family name

Institution

Department

Street and number

Post-code and town

Phone number/ Fax number

Address

City - State - Post-code

Tel. Fax

e-mail

Date

Signature

Membership fees for 2010: € 125,00 for Full Members

Membership fees may be paid as follows:

by bank transfer free of charge credited to the F.I.O. W.F.O.O.T., account

BANCA CARIGE - Agenzia 2 - BRESCIA - ITALIA

IBAN IT35 K061 7511 2020 0000 0624 780 - BIC CRGEITGG542

Please do not forget to indicate your name on the bank transfer.

Dr. Matteo Bonetti

W.F.O.O.T. (World Federation of Oxygen - Ozone Therapy)

Please type or write in capital letters.

Please return this form to:

CENTRAL OFFICE

X Ray Service s.r.l.

Via Oberdan, 140

25128 Brescia (BS)

Tel.: 030/3701312

Fax: 030/3387728

mail: info@xrayservice.it

INTERNATIONAL JOURNAL OF OZONE THERAPY

The Official Journal of the WFOT - World Federation of Oxygen-Ozone Therapy,
FIO - Italian Federation of Ozone Therapy, ACEOOT - Spanish Association of Ozone Therapy,
Hellenic, Indian, Slovach and Chinese National Societies

Please enter my subscription to International Journal of Ozone Therapy
www.centauro.it

- 1 year Italy: Euro 50,00
 1 year European Countries: Euro 55,00
 1 year non-European Countries (air mail): Euro 60,00

I enclose a cheque/money order payable to Centauro s.r.l.

Swift transfer Centauro s.r.l., Unicredit Banca - Swift code: UNCRITB1BA2 -
IBAN code: IT 74 D 02008 02435 000002933096

Please charge my Credit Card:

Visa Diners Master Card Carta Si Amex

No. _____ Expiry date _____

Name (please print)

Address

City - State - Post code

e-mail Tel. Fax

Date Signature

Please send invoice to

Please return this form to:

CENTAURO SRL
Via del Pratello, 8
I-40122 Bologna - Italy
Tel. 051.227634 - Fax 051.220099

Dear subscribers - In relation to the provisions of DLgs 196/03, we assure you that your personal information (name, surname, qualification, profession and address) currently stored in our database shall be used solely for the purpose of sending business letters and notices from publisher to subscriber. In according with clause 7 of DLgs 196/03, you are entitled to refuse to authorise any use of the information in our possession for purposes other than those prescribed by law.



Federazione Italiana di Ossigeno-Ozonoterapia

www.webfio.it

Al Presidente della FIO

Il sottoscritto/a

Codice Fiscale

Residente in via

CAP Città

e-mail Telefono Fax

Chiede di essere iscritto alla FIO - Federazione Italiana di Ossigeno-Ozonoterapia.

Allega un breve curriculum vitae (una pagina)

Data Firma

Mi impegno al versamento della quota sociale annua di 125,00 €.

Di cui 85,00 € come iscrizione alla FIO e 40,00 € come abbonamento alla Rivista "International Journal of Ozone Therapy", organo ufficiale della FIO, Banca Carige agenzia 2 di Brescia,

IBAN: IT 35K0617511202000000624780, oppure inviare con bollettino postale

C/C nr. 43650316, intestato a F.I.O. (Federazione Italiana di Ossigeno-Ozonoterapia).

Dr Matteo Bonetti
Segretario FIO

Presso: X-Ray Service Srl

Sede amministrativa: Via Leonardo da Vinci, 20 - 25122 Brescia

Sede operativa: Via Oberdan, 140 - 25128 Brescia

Tel.: 030.3701312/030.3387729 - Fax: 030.3387728

e-mail: segreteriafio@webfio.it

Comunicazione all'Abbonato - In relazione a quanto disposto dall'art. 10 della L. n. 675/1996, Le assicuriamo che i Suoi dati (nome, cognome, titolo di studio, attività svolta e indirizzo), presenti nel nostro archivio informatico, verranno utilizzati esclusivamente per l'invio di lettere commerciali e avvisi promozionali inerenti al rapporto editore-abbonato. Ai sensi dell'art. 13 della L. n. 675/1996, Lei potrà opporsi all'utilizzo dei dati in nostro possesso se trattati in maniera difforme a quanto disposto dalla legge

Via Oberdan, 140 - 25128 Brescia • C.F.: 98109470173 • Tel. 030 3701312 • Fax 030 3387728

FEDERAZIONE ITALIANA DI OSSIGENO-OZONOTERAPIA

Date.....

Re: association membership fee

Dear Colleague,

This is a reminder that the Association *membership* fee for 2010 is € 125,00, inclusive of a subscription to the International Journal of Ozone Therapy, *payment by bank draft to Banca Carige - agenzia 2 - Brescia, Italia*

IBAN: IT 35 K 06175 11202 000000624780 SWIFT Code: CRGEITGG542, or by credit card

Thank you in advance for your payment.

Yours sincerely,

Dr Matteo Bonetti
FIO Secretary

Objeto: cuota de asociación

Estimado Colega,

quería recordarte que *la cuota* de asociación por *el año 2010 es de € 125,00*, la que incluye la subscripción a International Journal of Ozone Therapy,

con un pago en la Banca Carige - agenzia 2 - Brescia, Italia

IBAN: IT 35 K 06175 11202 000000624780 Codigo SWIFT: CRGEITGG542, o con la tarjeta de crédito.

Te agradezco desde ahora por el pago de la cuota.

Cordialmente

Dr Matteo Bonetti
Segreteria FIO

Oggetto: quota associativa

Caro Collega,

desidero ricordarti che *la quota sociale* della FIO è per il 2010 di € 125,00,

comprensiva dell'abbonamento alla rivista International Journal of Ozone Therapy, *con un bonifico alla Banca Carige - agenzia 2 - Brescia, Italia*

IBAN: IT 35 K 06175 11202 000000624780 o con carta di credito.

oppure inviare con bollettino postale: c/c nr. 43650316, intestato a F.I.O.

(Federazione Italiana di Ossigeno-Ozonoterapia)

Ti ringrazio fin da ora per il pagamento.

Cordialmente

Dr Matteo Bonetti
Segreteria FIO

WFOOT WORLD FEDERATION OXYGEN-OZONE THERAPY

V.J. Kumar
President

Cosma F. Andreula, *Vice-President*
Matteo Bonetti, *Secretary General*
Alessandro Fontana, *Treasurer*

Advisors: E. Iliakis, J. Baeza Noci, Byung Chan Jeon,
X. He, O. Pepa, X.F. Vilasuso, G. Barco,
J.C. De Lucas-García, M. Leonardi, J. Vilitelka

INTERNATIONAL JOURNAL OF OZONE THERAPY

The Official Journal of the WFOOT - World Federation of Oxygen-Ozone Therapy, FIO - Italian Federation of Ozone Therapy, ACEOOT - Spanish Association of Ozone Therapy, Hellenic, Indian, Slovach and Chinese National Societies

INTERNATIONAL REVIEWERS

Ramiro Alvarado (Bolivia), G. Barco (President Eumedica),
Velio Bocci (Italy), Marco Brayda-Bruno (Italy),
J.C. De Lucas-García (Spain), Eugenio Genovese (Italy),
Stergios Ikonomidis (Greece), Emmanuel Iliakis (Greece),
Byung Chan Jeon (South Korea), Vijay Sheel Kumar (India),
Mario Muto (Italy), José Baeza Noci (Spain),
Gianni Pellicanò (Italy), Osvaldo Pepa (Argentina),
Vassilio Politis (Greece), Rafik Raslan (Dubai),
Hector Salgado (Spain), X.F. Vilasuso (U.S.A.),
Sergio Viti Paganelli (Venezuela),
Wally Kos (Australia), Juray Vilitelka (Slovakia),
He Xiaofeng (China)

SCIENTIFIC EDITOR

Matteo Bonetti
Servizio di Neuroradiologia - Istituto Clinico Città di Brescia
via Gualla, 15 - 25100 Brescia
Tel. ..39/030/3710311 - Fax ..39/030/3710370
e-mail: matbon@numerica.it

PUBLISHING STAFF

PUBLISHER
NICOLA LEONARDI
Direttore responsabile
Centauro srl
Via del Pratello, 8
I-40139 Bologna, Italy
Tel: ..39/051/227634
Fax: ..39/051/220099
e-mail: nicola.leonardi@centauro.it

LANGUAGE EDITOR

Anne Collins
e-mail: collins@iol.it

MANAGING EDITOR

Elisabetta Madrigali
e-mail: elisabetta@centauro.it

ADVERTISING

Serena Preti
e-mail: serena.preti@centauro.it

PRINTED BY:
Grafiche dell'Artiere - Bentivoglio (BO) Italy

INSTRUCTIONS FOR AUTHORS

First formulated in 2002 - Revised in June, 2010

The International Journal of Ozone Therapy (OJOT) is a clinical practice journal documenting the current state of ozone therapy practice worldwide. **OJOT** publishes original clinical observations, descriptions of new techniques or procedures, case reports, and articles on the ethical and social aspects of related health care. Submissions suitable for the Journal include observational studies, clinical trials, epidemiological work, reports on health services and outcomes, and advances in applied (translational) and/or basic research.

The instructions for submission of articles to **OJOT** follow the *Uniform Requirements for Manuscripts Submitted to Biomedical Journals of the International Committee of Medical Journal Editors (ICMJE, <http://www.icmje.org>)*, if not otherwise indicated below.

Research and Publication Ethics

For the policies on research and publication ethics not stated in these instructions, the Guidelines on *Good Publication Practice* issued by the Committee on Publication Ethics should be applied.

1. *Author responsibility for originality:* Each author acknowledges responsibility for the integrity of the manuscript, assures the originality of the paper, and guarantees that submitted manuscripts do not contain previously published material and are not under consideration for publication elsewhere. If the submitted manuscript builds on or includes parts of previously published articles, authors are encouraged to enclose copies of the articles with the new submission. The Editor reserves the right to request the original data obtained in the investigation.

2. *Registration of clinical trial research:* Any research that includes clinical trials should be registered with the primary national clinical trial registration authority accredited by WHO (<http://www.who.int/ictrp/network/primary/en/index.html>) or ICMJE.

3. *Disclosure statement:* This is not intended to prevent authors with potential conflicts of interest from contributing to **OJOT**. Rather, the Journal will place on record any relationship that may exist with disclosed, or competing, products or firms. Disclosed information will be held in confidence during the review process and the Editors will examine any printed disclosure accompanying a published article. Authors are responsible for notifying the Journal of financial arrangements including, but not limited to, agreements for research support including provision of equipment or materials, membership of speaker bureaus, consulting fees, or ownership interests. It is important that disclosure statements be updated promptly to reflect any new relationships that arise after initial submission of the manuscript. If the study is supported by a commercial sponsor, the authors must document the input of the sponsoring agency in study design, data collection, analysis of results, interpretation of data, and report writing. It is important to specify whether the sponsors could have suppressed or influenced publication if the results were negative or detrimental to the product they produce. Authors should also state if the company was involved in the original study design, the collection and monitoring of data, analysis and/or interpretation, and/or the writing and approval of the report.

4. *Statement of human and animal rights:* Manuscripts involving examination of volunteers and patients must include a statement that the study protocol has been approved by an institutional review board (IRB) and that the subjects gave informed consent, thus meeting the standards of the Declaration of Helsinki (revised version) developed by the World Medical Association as a statement of ethical principles for medical research involving human subjects, including research on identifiable hu-



For information: Centauro s.r.l.
Via del Pratello, 8 - I-40122 Bologna
Tel: ..39/051/227634 - Fax: ..39/051/220099
e-mail: serena.preti@centauro.it
www.centauro.it
SINGLE ISSUE: Euro 30,00
Back Issue: 50% surcharge
Reg. Trib. di Bologna n. 7159 del 20-09-2001

man material and data (<http://www.wma.net>). With animal subjects, research should be performed based on a National or Institutional Guide for the Care and Use of Laboratory Animals, and ethical treatment of all experimental animals is expected throughout all experiments.

5. **Duplicate/Redundant publication:** **OJOT** only accepts manuscripts describing original research. The editorial office of the **OJOT** does not accept duplicate submission or redundant publication. Redundant (or duplicate) publication is publication of a paper that overlaps substantially with a paper already published in print or electronic media as defined by the updated ICMJE guidelines that cover allegations of scientific misconduct. When submitting a paper, authors should make a full statement to the Editor on all submissions and previous reports that might be regarded as redundant publication of the same or similar work. If authors believe that their manuscript may be considered redundant, they should address this issue in a letter to the Editor accompanying the submission. The authors should also explain in the letter how their report overlaps already published material, or how it differs. Copies of such published material should be included with the submitted paper to help the Editor examine the possibility of redundant publication. If redundant publication is attempted without such notification, authors should expect editorial action to be taken. At the very least, rejection of the manuscript may be expected.

6. **Authorship:** The Editor considers authorship to belong to those persons who accept intellectual and public responsibility for the statements made and results reported. By submitting a manuscript for publication, each author acknowledges having made a substantial contribution to the concept and design of the study, the analysis and interpretation of the results, and the writing of the paper, in addition to having approved the final submitted version. Authorship should not be attributed to Departmental Chairs not directly involved in the study, to physicians or technicians who provided routine services, or to technical advisors. A group study should carry the group name and reference contributing authors in the Acknowledgments.

Peer Review Process

Papers are accepted on the understanding that they are subject to peer review, editorial revision, and, in some cases, comment by the Editor. Manuscripts are examined by independent peer reviewers. Articles and other material published in the Journal represent the opinions of the authors and should not be construed to reflect the opinions of the publisher.

Language

The official language of **OJOT** is English. Most papers in **OJOT** have been written by non-native English speakers, and they will also be read by many non-native speakers. For the purposes of clarity, it is strongly recommended that authors not fluent in English have their manuscript professionally edited for English usage prior to submission. A professional editor will improve the English to ensure that author meaning is clear and to identify problems that require author review. Authors are invited to contact the **OJOT** language editor Anne Collins (collins@iol.it) for referral to professional English language editing services. Authors should make contact with and arrange payment to their chosen editing service directly. Please note that the use of such a service is at the author's own expense and risk and does not guarantee that the article will be accepted for publication. Centauro Publishers does not receive any commission or other benefit from editing services. Accordingly, Centauro Publishers neither endorses nor accepts any responsibility or liability for such editing services.

Copyright and Purchase of Offprints

All articles published in **OJOT** are protected by copyright, which embraces the exclusive right to reproduce and distribute the article (e.g., as offprints), as well as all translation rights. No material published in the Journal may be reproduced without written permission from the publisher. **OJOT** offers authors a complimentary hard copy and .pdf file of the issue in which their article is published, for personal use. To purchase offprints of articles published by **OJOT** please contact Centauro Srl for a quotation at serena.preti@centauro.it.

Publication Types

Original Research Articles are full-length research papers, which are favored by **OJOT**. Articles cover topics relevant to clinical studies, and may include both basic and experimental work.

Review Articles are comprehensive, state-of-the-art papers focusing on important clinical problems and should address a specific topic in a scholarly manner. Such articles may be invited by the Editor or may be unsolicited reviews.

Case Reports/Technical Notes should be unusually educational and medically important. Although **OJOT** preferentially encourages submission of full-length Original Research Articles, the Journal will consider publication of a limited number of concise Case Reports and Technical Notes.

Editorials are usually invited by the Editor.

Editorial Comments are specific comments on the articles published in **OJOT** and are usually invited by the Editor.

Letters to the Editor contain constructive comments or criticism of a specific paper published by **OJOT**. Letters dealing with subjects of general interest within the field of **OJOT**, or personal opinions on a specific subject within the ambit of **OJOT**, may also be accepted.

All papers should be sent to the Scientific Editor Dr. Matteo Bonetti: matbon@numerica.it

Manuscript Preparation

Original research should be organized in the customary format, as described below. The text of the manuscript should be submitted as a single document with the following sections (in order):

1. **Author Information Page** (First page) - Full title of the article, authors' names, highest academic degree earned by each author, authors' affiliations, name and complete address for correspondence, address for reprints if different from address for correspondence, fax number, telephone number, and e-mail address.

2. **Acknowledgments and Funding Page** (Second page) - The Acknowledgments section lists all funding sources for the research of the study, and details substantive contributions of individuals. The authors must reveal all possible Conflicts of Interest/Disclosures.

3. **Title Page** (Third page) - The full title, itemized list of the number of tables, the number and types (color or black-and-white) of figures, and three-to-five key words for use as indexing terms should be included. Appropriate key words should be selected from the Medical Subject Heading. The word count of the text should be specified.

4. **Summary** - A summary of up to 250 words should summarize the problems presented and describe the studies undertaken, results and conclusions. Since the abstract must be explicative, the abbreviations must be reduced to a minimum and explained. References should not be cited in the abstract.

5. **Text** - Typical main headings include Introduction, Materials and Methods, Results, Discussion, and Conclusions. Abbreviations must be defined at first mention in the text, tables and figures. The complete names and short addresses of manufacturers of any equipment used in Materials and Methods must be supplied. If animals are used in experiments, state the species, the strain, the number of animals used, and any other pertinent descriptive characteristics. If human subjects or patients are employed, provide a table with relevant characteristics. When describing surgical or neurointerventional procedures on animals, identify the pre-anesthetic and anesthetic agents used, and state the amount or concentration and the route and frequency of administration of each agent. Generic names of drugs must be given. Manuscripts that describe studies on humans must indicate that the study was approved by an Institutional Review Committee and that all subjects gave informed consent. Reports of studies on both animals and humans must indicate that all procedures followed were in accordance with institutional guidelines.

6. **References** - Citations should be listed in order of appearance in the text, and between square brackets []. References must be listed at the end of the text in the order of citation. Journal titles should be abbreviated according to Index Medicus. For citation rules not specified here, authors should refer to the *NLM Style Guide for Authors, Editors, and Publishers* (<http://www.nlm.nih.gov/citingmedicine>). All references must be checked by the author(s). *Journal article:* Laredo JD, Bard M. Thoracic spine: percutaneous trephine biopsy. *Radiology*. 1986; 160: 485-489.

Journal article if the number of authors is more than six: list the first three authors followed by et al.

Geibprasert S, Krings T, Pereira V, et al. Clinical characteristics of dural arteriovenous shunts in 446 patients of three different ethnicities. *Intervent Neuroradiol*. 2009; 15: 395-400.

Entire book: Valavanis A. *Medical radiology: interventional neuroradiology*. Heidelberg: Springer Verlag; 1993.

Part of book if number of authors is more than two:

Bonneville JF, Clarisse J, et al. Radiologie interventionnelle. In: Manelfe C ed. *Imagerie du rachis et de la moelle*. Paris: Vigot Editeur; 1989. p. 761-776.

7. **Tables** - Each table must be typed on a separate sheet and double-spaced, if possible. Tables should be numbered using the Arabic system, followed by a brief informative title. Use type of the same font and size as employed in the text. Include footnotes at the bottom of each table. Tables must be numbered in the order cited in the text. Tables should not duplicate data given in the text or figures.

8. **Figure Legends** - Provide figure legends on a separate sheet. Legends must be double-spaced, and figures must be numbered in the order cited in the text.

9. **Figures** - Figures should preferably be submitted online in **.tiff format** to: matbon@numerica.it. The time required to send files will vary depending on the number of figures, but image resolution **must not be reduced** to decrease transmission time. When labeling the figures, please ensure that the label corresponds to the figure number.

Digital images (originals or images acquired by a scanner) must meet the following criteria:

Black-and-white figures: Images must be acquired using the grey scale with a minimum resolution of 300 pixels per inch or 150 pixels per cm. Images must have a base of at least 8.1 cm for one item or a minimum base of 16.9 cm for several items.

Color figures: Images must be acquired using the full color CMYK method with a minimum resolution of 300 pixels per inch or 150 pixels per cm. Images must have a base of at least 8.1 cm for one item or a minimum base of 16.9 cm for several items. The RGB method is

recommended for video reproductions only, as the quality of printed figures is poor.

Images must be saved in **.tiff format**. Images in .jpg format are not acceptable as details tend to be lost upon scanning, even at high resolution. Image definition also depends on the enlargement factor. Thus, a large low-resolution image can be proportionally reduced (by 24%) for publication, thereby permitting optimal presentation in print. However, enlargement of a small high-resolution image will highlight all flaws, yielding a pixelated effect.

Do not submit figures already paged in Word, PowerPoint, or other documents, or images inserted in web pages. Such images are of low resolution and are unsuitable for printing.

Figures in .dcm (Dicom) format may be submitted as .dcm or .tiff files. The editorial office will process such images for printing.

Illustrations may be compressed using the StuffIt, Aladdin, or Zip programs. Do not label an image with arrows, numbers, or letters, but rather indicate on a duplicate copy or a sketch where such indications are desired. Authors are advised to refer to the Journal guidelines when formatting their work. Otherwise the editorial office may return a submission to the authors for improvement before any Editor is assigned.

If it is not possible to send figures via the Internet, images may be sent by express courier in one of the following formats:

Original x-ray films, slides, or glossy or opaque prints. Clearly indicate on the back of the image the first author's name and the number corresponding to the figure caption and citation in the text.

Figures may be submitted on a CD-ROM or DVD, copied in ISO9006 format legible on both PC and MAC. Floppy disks are not recommended. Clearly indicate on one side of the figure both the top of the figure and the figure number. Do not label the actual image with arrows, numbers, or letters, but rather indicate the top and figure number on a duplicate copy or on a sketch. Do not cut or attach figures with adhesive tape or use paper clips. If images are sent offline, thumbnails of the images should be uploaded as a submission item online, together with the manuscript, to allow the publishers to reserve space for the original high-resolution images. All material should be sent to the editorial office:

The International Journal of Ozone Therapy

Centauro S.R.L.

Via del Pratello, 8

I-40122 Bologna - Italy

Tel: +39.051.227634

Fax: +39.051.220099

E-mail: marco.leonardi@centauro.it

INTERNATIONAL JOURNAL OF OZONE THERAPY

Conflicts of Interest/Disclosures, Copyright Transfer Agreement, Financial Support, Exclusive Publication Statement, and Author Contribution Form

Manuscript No.: _____

Date: _____

With respect to the article entitled: _____

The authors certify that:

(Conflicts of Interest/Disclosures) The corresponding author must disclose all relevant financial, personal, and/or professional relationships with other people or organizations related to the subject of this article. Is there any actual or potential conflict of interest in the subject of this article?

No (___). If yes, please give details in the space below.

(Copyright Transfer Agreement) All rights, title, and interest in the manuscript, including copyright ownership, are transferred to Centauro Srl Publisher, Bologna. Authors reserve all proprietary rights (such as patent rights) other than copyright and the right to use parts of this article in their own subsequent work as soon as the article is accepted for publication. The article may not be published elsewhere without permission from the publisher.

(Financial Support) If the authors or their institution(s) is/are affiliated with any organization having a financial interest (including the provision of financial support) in the subject of this article, the authors should indicate (a) the name of the industry/institution providing support for the study, (b) the type of affiliation of the organization (stockholder, consultant, donor of honorarium, granting body, source of other financial or material support, provider of equipment, or other support category), and (c) that the authors had complete control of the data and information submitted for publication. Any other non-financial conflict of interest should also be disclosed to the Editor, with the understanding that the information may be published if deemed appropriate by the Editor.

No, I (We) do not have any conflict of interest related to this article (___).

If yes, (a) (_____) (b) (_____) (c) (_____).

(Exclusive Publication Statement) None of the material in this manuscript has been published previously or submitted for publication elsewhere prior to appearance in NRJ.

(Author Contribution Form) The corresponding author must certify that all authors participated in and contributed sufficiently to the conception and design of the work, and analysis of the data, as well as the writing of the manuscript, and assume public responsibility for the content of the paper. The corresponding author also certifies that all authors have reviewed the final version of the manuscript and approved it for publication.

Name (print) _____ Signature _____ Date _____

This signed statement must be received before the manuscript can be processed for publication.