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Ozone oxidative postconditioning ameliorates joint damage and decreases pro-inflammatory cytokine levels and oxidative stress in PG/PS-induced arthritis in rats



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ABSTRACT

Rheumatoid Arthritis (RA) is the most prevalent chronic condition present in ~1% of the adult population. Many pro-inflammatory mediators are increased in RA, including Reactive Oxygen Species such as nitric oxide NO, pro-inflammatory cytokines as tumor necrosis factor alpha (TNF- α), interleukin-1beta (IL-1 β) and other molecules. Ozone oxidative postconditioning has regulatory effects on some pathological targets associated with RA. Thus, the aim of this study was to investigate the efficacy of ozone therapy in PG/PS-induced arthritis in rats in point of joints inflammation and morphology. Moreover, cytokines, nitric oxide and oxidative stress levels in spleen homogenates were evaluated. Ozone treatment ameliorated joint damage, reduced TNF- α concentrations as well as TNF- α and IL-1 β mRNA levels. Besides, cellular redox balance, nitric oxide and fructolysine levels were reestablished after ozone oxidative postconditioning. It was concluded that pleiotropic ozone's effects clarify its therapeutic efficacy in RA. Decreasing inflammation and joint injury, reduction of pro-inflammatory cytokines, TNF- α and IL-1 β transcripts and re-establishment of cellular redox balance after ozone treatment were demonstrated.

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1. Introduction

The most prevalent of chronic conditions is Rheumatoid Arthritis (RA), which exists in ~1% of the adult population (Symmons et al., 2002). RA is a chronic inflammatory disease characterized by synovial inflammation and pannus formation which can lead to severe destruction of cartilage and bone. Several self-proteins have been suggested to be disease-driving autoantigens. Moreover the presence of autoantibodies to different proteins in sera of patients with RA enhances the strength of this hypothesis (Alivernini et al., 2008).

Much evidence from animal models of arthritis and human studies suggests that a Th1 mechanism is involved in inflammatory arthritis (Gaston, 1995). Many pro-inflammatory mediators are expressed in RA, including Reactive Oxygen Species such as

nitric oxide, pro-inflammatory cytokines as TNF- α and IL-1 β , proangiogenic molecules and others. However, evidence also suggests important roles for other cell types, such as mononuclear phagocytes. It has been demonstrated that significant changes occur in both T cells and mononuclear phagocytes during the development of SCW (streptococcal cell wall)-induced chronic inflammatory arthritis. These cellular changes were most evident in the spleen but also occurred in lymph nodes and peripheral blood (Kimpel et al., 2003).

Today, there is no direct cure for RA available; the main goals of treatment are therefore to ameliorate the symptoms of the disease (i.e., diminish pain and decrease inflammation and joint destruction). Different types of treatments are currently used as Non-steroidal anti-inflammatory drugs (NSAIDs), Disease-modifying antirheumatic drugs (DMARDs) and Biological agents such as anti-TNF- α (Gelderman et al., 2007).

Taking into account that medical ozone is a bioregulator which protects against damage by chronic oxidative stress through an oxidative pre/postconditioning mechanism (Leon et al., 1998), regulates nitric oxide levels (Ajamieh et al., 2004), p65 subunit

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of nuclear factor kappa B (NF- κ B), and tumor necrosis factor alpha (TNF- α) (Leon Fernandez et al., 2008), the aim of this work was to investigate the effects of ozone therapy on joint swelling, pro-inflammatory cytokines and oxidative stress in the experimental chronic polyarthritis induced by i.p. injection of bacterial cell wall peptidoglycan-polysaccharide (PG/PS) *in vivo*.

2. Material and methods

2.1. Animals

Adult female Lewis rats (100–120 g) were used for these studies. Rats were maintained in air filtered and temperature conditioned (20–22 °C) room with a relative humidity of 50–52%. Rats were fed with standard commercial pellets and water *ad libitum*. All procedures were performed as approved by the Institutional review board (Scientific and Ethics Committees of the Institution) and in accordance with the European Union Guidelines for animal experimentation.

2.2. Chemicals

Ozone (O₃) was generated by an OZOMED equipment manufactured by the Ozone Research Center (Cuba) and was administered into intra-articular space. Ozone was obtained from medical grade oxygen, was used immediately as generated and it represented only about 3% of the gas (O₂+O₃) mixture. The ozone concentration was measured by using a built-in UV spectrophotometer set at 254 nm (accuracy, 0.002 A at 1 A, repeatability 0.001 A and calibrated with internal standard). The ozone dose was the product of the ozone concentration [expressed as mg/l by the gas (O₂+O₃) volume (l)]. By knowing the body weight of the rat, the ozone dose was calculated. Ozone concentration was 20 μ g/ml and the total dose administered was 80 μ g/kg.

The 10S fraction of peptidoglycan-polysaccharide (PG-PS) was purchased from Lee Laboratories (Grayson, GA). The material was briefly sonicated before use. All other chemicals and reagents used in this study were purchased from Sigma (St. Louis, MO) unless specified otherwise.

2.3. Induction of rat arthritis by PG-PS injection

The standard protocol for SCW-induced arthritis was followed as it was described by the manufacturer. Briefly, rats were given a single intraperitoneal (i.p.) injection (0.1 ml) of PG-PS (Lee Laboratories, Grayson, GA, 15 μ g rhamnose/g body weight). Control animals were injected with an equal volume of saline. Rats were observed daily, and the development of arthritis was assessed by objective and histopathological criteria.

2.4. Treatment schedule

The protocol consisted of four experimental groups ($n=20$). Group 1 ($n=5$): Control (–) only received the needle stress into articular space 3 times/week; Group 2 ($n=5$) rats received PG/PS; Group 3 ($n=5$) as group 2 but after 10 days of PG/PS administration rats received 0.2 ml of ozone/oxygen mixture which was administered into articular space 3 times/week (3.5 weeks). Hind paws were selected by the treatments. 25G \times 5/8" needles were used and their lengths were adjusted through space bar to 3 mm; Group 4 ($n=5$) as Group 3 but ozone treatment was substituted by oxygen.

2.5. Evaluation of arthritis

Thickness ankle of each rat (maximal lateral) was achieved with a caliper in order to determine the baseline in both left and right ankles. Each ankle was measured 3 times and averaged.

After PG/PS injection the response of each rat was monitored every day for the first 10 days, then 3 times/week during 24 days (end of the experiment). Likewise, a reading of the response consisted of 3 measurements with a caliper per ankle. Each group of rat ankle measurements were averaged and recorded as one data point to produce a graphical representation of the data.

Joint swelling was scored (arthritis index) according to the standardized method by an experienced observer (Kimpel et al., 2003). Briefly, a score of 0–4 was assigned as follows: 0, no evidence of hyperemia and/or inflammation; 1, hyperemia with little or no paw swelling, 2, swelling confined predominantly to the ankle region, with modest hyperemia, 3, increased paw swelling and hyperemia of the ankle and metatarsal regions, and 4, maximal paw swelling and hyperemia involving the ankle, metatarsal, and tarsal regions.

2.6. Sample preparations

At the end of the experiment rats were killed by diethyl ether anesthesia. Afterwards the spleen was promptly removed for biochemical studies. Spleen homogenates were obtained using a tissue homogenizer Edmund Bühler at 4 °C. The homogenates were prepared using a 50 mM KCl/histidine buffer pH 7.4, 1:10 (w/v) and were spun down with a Sigma Centrifuge 2K15, at 4 °C and 8500 \times g for 20 min. Supernatants were taken for biochemical determinations.

2.7. Biochemical determinations

The biochemical parameters were evaluated in the supernatants of spleen homogenates 30 days after PG/PS-induced arthritis and 24 h after the last treatment with ozone or oxygen. The different parameters were determined by spectrophotometric methods using an Ultrospect Plus Spectrophotometer from Pharmacia LKB. Concentration of tumor necrosis factor alpha (TNF- α) in spleen homogenates was determined by a specific commercial ELISA kit (Quantikine, R&D Systems, Minneapolis, MN, USA) according to the recommendations of the manufacturers. The minimum detectable dose of TNF- α was less than 5 pg/ml. Nitrite/nitrate levels were determined by the Griess reaction by first converting nitrates to nitrites using nitrate reductase (Boehringer Mannheim Italy SpA, Milan, Italy). Then the Griess reagent (1% sulfanilamide, 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride in 0.25% phosphoric acid) was added (Granger et al., 1995). Superoxide dismutase activity in the spleen homogenates was determined by measuring the inhibition of pyrogallol auto-oxidation (Boehringer_Mannheim, 1987) where a unit of activity was defined as the amount of enzyme required to inhibit the rate of pyrogallol autooxidation by 50%. Catalase activity was measured by following the decomposition of hydrogen peroxide at 240 nm at 10 s intervals for 1 min (Boehringer_Mannheim, 1987). Relative fructolysine content (Amadori's product of glycated protein) was measured by reduction of the redox indicator nitrobluetetrazolium (NBT) at 530 nm (Thome et al., 1996).

2.8. Analysis of TNF- α and IL-1 β by quantitative PCR (qPCR)

Total RNA in spleen homogenate was extracted as per manufacturer's instructions of the commercial kit RNeasy Plus Micro (Qiagen, United Kingdom). Briefly, cDNA was synthesized from total RNA as commercial kit Quantitect Reverse Transcription

(Qiagen, United Kingdom). In the experiment was used GAPDH as reference gene like inside control in order to guarantee the same amount of cDNA in all samples. Contaminant genomic DNA was removed as manufacturer's instructions. Nevertheless, controls of the experiment without Reverse Transcriptase enzyme were included in order to discard genomic DNA presence.

Primer oligonucleotides were delineated as cDNA sequences available in GenBank (<http://www.ncbi.nlm.nih.gov>) and primer sequences were the following: TNF- α , 5'-TGCCTATGCTCAGCCTCTTC-3' y 5'-GAGGCCATTTGGAACTTCT-3'; IL-1 β , 5'-AAGTTGACGGACCCAAAA-GAT-3' y 5'-TGTTGATGTGCTGCTGCGA-3'; GAPDH, 5'-CGGAGTCAACG-GATTGGTCGTAT-3' y 5'-AGCCTTCTCCATGGTGGTAA GAC-3'.

mRNA levels correspondents of TNF- α , IL-1 β and GAPDH genes were carried out in Light Cycler (Bio Rad Inc., EU) equipment. Each sample was analyzed in triplicated. Reference genes were selected according to the computer program GeNorm. Quantification was achieved by the $\Delta\Delta$ CT method and the expression levels were normalized against reference gene GAPDH. REST program was used in the statistical analysis and a statistical meaning of $P < 0.05$ was assumed.

2.9. Histological study

Rats' hind paws were fixed in formaldehyde at 4% during 24 h. Tissue was decalcified with formic acid and sodium citrate.

2.9.1. Sample processing

After dehydration and xylol treatment samples were included in paraffin wax with a processor tissue equipment (Sakura, Model RH-12EP-2). After paraffin inclusion, more than five cuts/sample using a micrometer (Leica Model RM2135, Meyer Instruments, Houston, TX, USA) with thickness of 5 mm were made.

H&E and Masson's Trichrome stains were used. Visualization and image capture was made through a Digital Chamber DP72 (Olympus; Center Valley, PA) matched on Olympus Microscope BX51. All histological study was achieved to double-blind by 2 different experts.

2.10. Statistical analysis

The OUTLIERS preliminary test for detection of error values was initially applied for statistical analysis. Afterward, the ANOVA method (single way) was used followed by the homogeneity variance test (Bartlett-Box). In addition, a multiple comparison test was used (Duncan test). Data were expressed as the mean \pm standard deviation of 5 animals. The level of statistical significance employed was at least $P < 0.05$ for all experiment.

3. Results

Fig. 1 shows Total Arthritis Index in each experimental group. Ozone treatment was able to ameliorate the joint swelling induced by PG/PS in comparison with PG/PS and PG/PS+Oxygen groups which were not different at the end of the experiment. After 4 ozone treatments a decrease of arthritis index was observed as well as a significant difference with regard to PG/PS and PG/PS +Oxygen at the end of the experiment. Similar picture was demonstrated when rat's ankle thickness was evaluated (Fig. 2).

Histological results are shown in Fig. 3. (A) Control (-), without alterations (B) PG/PS showed a proliferation of angioblast and new capillaries were observed. (C) PG/PS+Ozone displayed a normal morphology. (D) and (E) PG/PS+Oxygen exhibited the higher damage with hypertrophy and angiogenesis in the synovial membrane.

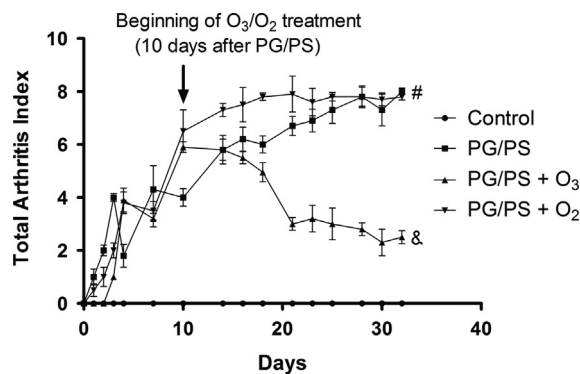


Fig. 1. Effects of ozone/oxygen treatments on Total Arthritis Index (joint swelling) induced by PG/PS. Four experimental groups were studied. Group 1 ($n=5$), received the needle's stress into articular space 3 times/week; Group 2 ($n=5$) rats received 0.1 ml of PG/PS (15 μ g rhamnose/g body weight); by i.p.; Group 3 ($n=5$), as group 2 but after 10 days of PG/PS administration rats received 0.2 ml of ozone/oxygen mixture (20 μ g/ml) into articular space 3 times/week (3.5 weeks); Group 4 ($n=5$), as group 3 but ozone was substituted by oxygen (26 mg/ml). Evaluation of each hind paw was made according to Section 2. Data represent the mean of three different independent experiments. $^{\&}$ $P < 0.05$ with respect to PG/PS, PG/PS+O₂ and control groups. $^{\#}$ $P < 0.01$ with respect to control for PG/PS and PG/PS+O₂ groups.

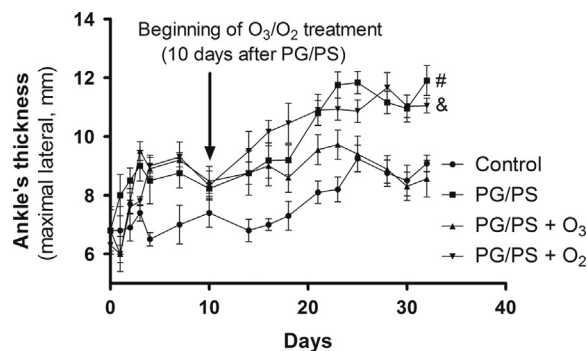


Fig. 2. Ankle's thickness of hind paws of Lewis rats ($n=5$) during the experiment after PG/PS-induced arthritis and the effects of ozone/oxygen treatments. Four experimental groups were studied. Group 1 ($n=5$), received the needle's stress into articular space 3 times/week; Group 2 ($n=5$) rats received 0.1 ml of PG/PS (15 μ g rhamnose/g body weight); by i.p.; Group 3 ($n=5$), as group 2 but after 10 days of PG/PS administration rats received 0.2 ml of ozone/oxygen mixture (0.7 mg/kg) into articular space 3 times/week (3.5 weeks); Group 4 ($n=5$), as group 3 but ozone was substituted by oxygen (26 mg/ml). Data represent the mean of three different independent experiments. $^{\&}$ and $^{\#}$ $P < 0.05$ with respect to Control and PG/PS+O₃ groups.

Fig. 4(A,B) shows TNF- α and nitric oxide (NO x) concentrations in the experimental groups. PG/PS+Ozone ameliorated TNF- α levels with regard to PG/PS group while PG/PS+Oxygen displayed the higher concentration since it increased ($P < 0.05$) in comparison with PG/PS group. Ozone treatment reestablished nitric oxide levels comparable to Control (-) ($P > 0.05$). PG/PS exhibited higher concentration of NO and PG/PS+Oxygen showed an increase of this free radical with regard to Control (-) and PG/PS+Ozone groups.

Fig. 5 shows mRNA of TNF- α and IL-1 β in spleen homogenates. Ozone treatment decreased ($P < 0.05$) both pro-inflammatory transcripts with regard to PG/PS and PG/PS+Oxygen groups.

Oxidative stress markers are shown in Table 1. SOD (scavenger of superoxide radicals) was not different to Control (-) group whereas PG/PS and PG/PS+Oxygen groups showed a decrease ($P < 0.05$) of this antioxidant enzyme. Catalase activity increased in the group treated with ozone in comparison with the rest of the experimental groups. Fructolysine was regulated by ozone treatment and its levels were similar to Control (-) ($P > 0.05$) whereas PG/PS and PG/PS+Oxygen groups increased the glycation proteins

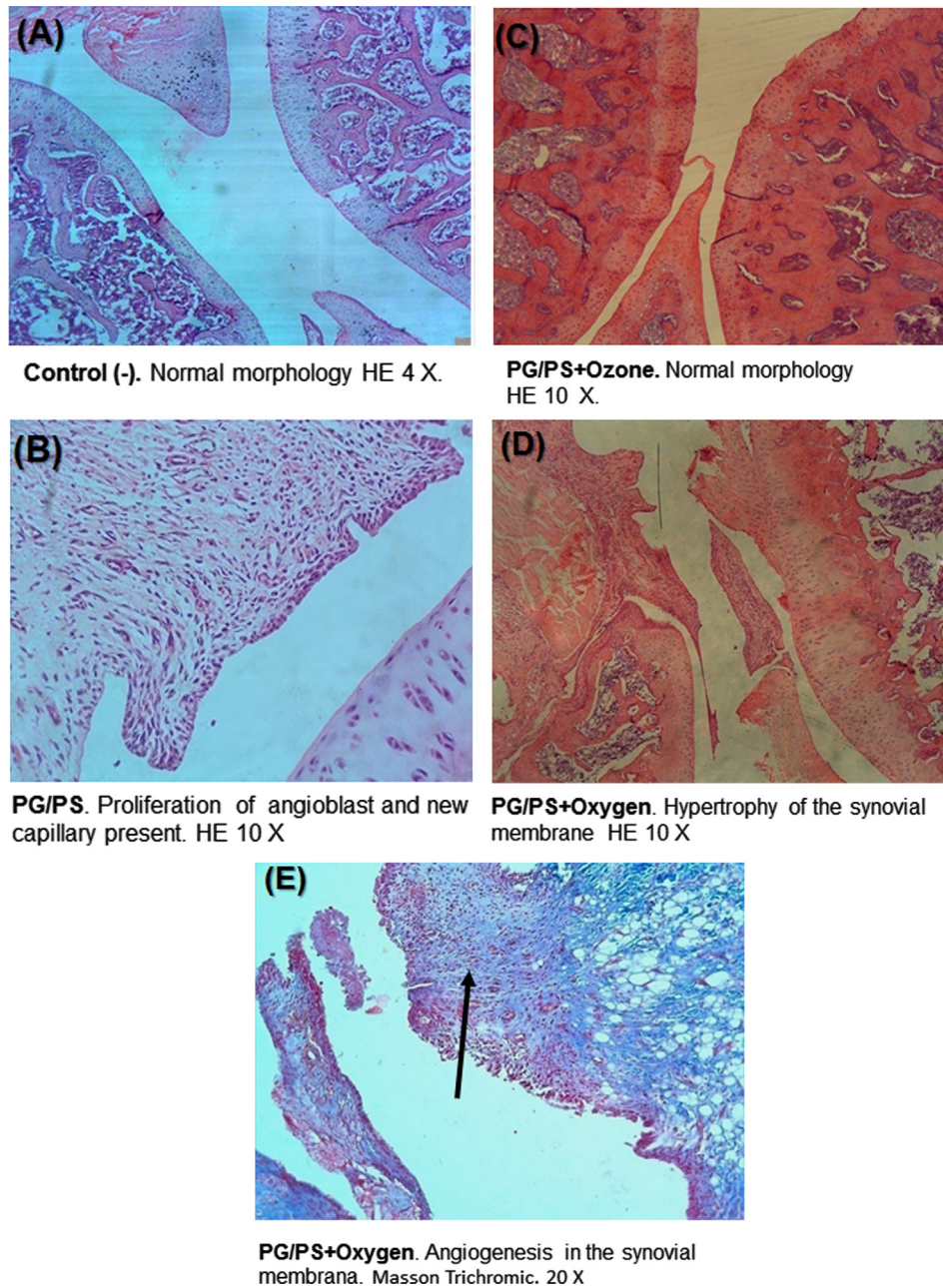


Fig. 3. Histological results of the experimental groups in PG/PS-induced arthritis and treated with ozone/oxygen.

and they were not different. Although CAT/SOD relationship (a redox index) increased in comparison with Control (-) it decreased ($P < 0.05$) with regard to PG/PS and PG/PS+Oxygen.

4. Discussion

The spleen, a secondary lymphoid organ, is a major site of immune surveillance, antigen recognition, activation, and clonal proliferation. It seems to play an important pathogenic role in RA due to splenectomy ameliorate joint swelling and decrease cytokines and NO levels as well as other molecules released during RA (Kimpel et al., 2003).

Ozone treatment decreased Total Arthritis Index as well as the ankle's thickness of hind paws of Lewis rats (Figs. 1 and 2) pointing at its beneficial effects in PG/PS-induced arthritis whereas an increase of injury in oxygen group (ozone's vehicle) was observed.

These results were in line with histological studies in which normal morphology in PG/PS+Ozone group was observed. In contrast, PG/PS and PG/PS+Oxygen groups showed proliferation, hypertrophy and angiogenesis (Fig. 3).

TNF- α is a pro-inflammatory cytokine, activating the NF- κ B pathway, leading to a downstream cascade of other pro-inflammatory cytokines (Karouzakis et al., 2006). Moreover, it is known to increase mitochondrial Reactive Oxygen Species (ROS) production (Woo et al., 2000). Current targeted biologic therapies, including anti-TNF- α inhibitors result in greater disease improvement and prevention of joint erosion, although clinical studies on the efficacy of TNF- α blocking agents clearly show that about 40% of patients receiving this therapy are non-responders (Biniacka et al., 2011).

Ozone reduced TNF- α concentrations in spleen homogenates ($P < 0.05$) with regard to PG/PS and PG/PS+Oxygen (Fig. 4). This cytokine is associated with the injury to joint and bone. Ozone's

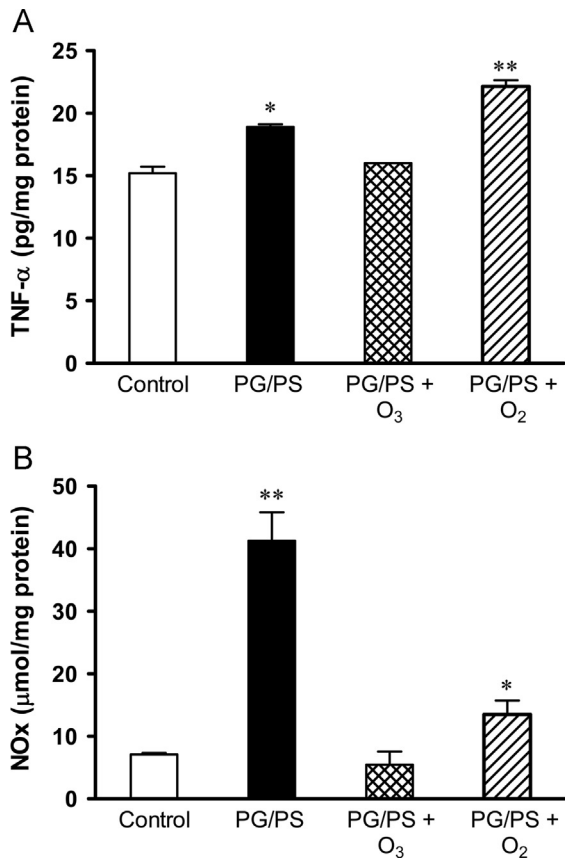


Fig. 4. (A), TNF- α and (B) nitric oxide (NO) concentrations in PG/PS-induced arthritis in rats (spleen homogenates). Four experimental groups were studied. Group 1 ($n=5$), received the needle's stress into articular space 3 times/week; Group 2 ($n=5$) rats received 0.1 ml of PG/PS (15 μ g rhamnose/g body weight); by i.p.; Group 3 ($n=5$), as group 2 but after 10 days of PG/PS administration rats received 0.2 ml of ozone/oxygen mixture (20 μ g/ml) into articular space 3 times/week (3.5 weeks); Group 4 ($n=5$), as group 3 but ozone was substituted by oxygen (26 mg/ml). Data represent the mean of three different independent experiments. * $P < 0.05$ and ** $P < 0.01$ with respect to Control.

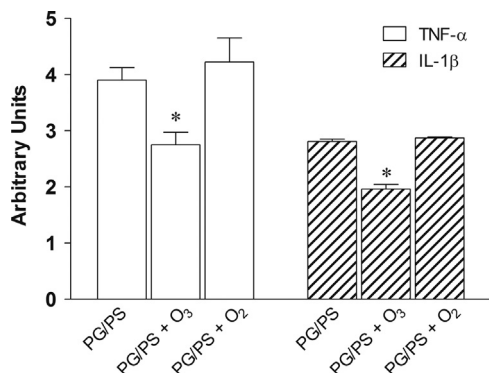


Fig. 5. mRNA levels of IL-1 β and TNF- α in rat's spleen homogenates from PG/PS induced-arthritis. Four experimental groups were studied. Group 1 ($n=5$), received the needle's stress into articular space 3 times/week; Group 2 ($n=5$) rats received 0.1 ml of PG/PS (15 μ g rhamnose/g body weight); by i.p.; Group 3 ($n=5$), as group 2 but after 10 days of PG/PS administration rats received 0.2 ml of ozone/oxygen mixture (20 μ g/ml) into articular space 3 times/week (3.5 weeks); Group 4 ($n=5$), as group 3 but ozone was substituted by oxygen (26 mg/ml). Data represent the mean of three different independent experiments. * $P < 0.05$ with respect to Control.

protective effects may be a consequence of ozone's ability to regulate 65 subunit NF- κ B as it was demonstrated in liver ischemia/reperfusion injury (Leon Fernandez et al., 2008). Increase

of TNF- α in PG/PS and PG/PS+Oxygen groups was in line with damage observed in histological studies (Fig. 3) underlining the pathological role of this cytokine in RA.

When mRNA levels of TNF- α and IL-1 β were determined in spleen homogenates it was observed that ozone treatment reduced ($P < 0.05$) the concentration of these transcripts in comparison with PG/PS and PG/PS+Oxygen groups (Fig. 5). Both cytokines are generated when NF- κ B is activated but at the same time TNF- α and IL-1 β are activators of NF- κ B giving rise to vicious circle which perpetuate the chronic inflammatory process therefore these results suggest that ozone treatment contribute to break noxious NF- κ B pathway.

Ozone efficacy in RA not only may be explained through its actions on cytokines but also besides ozone therapy is able to reestablish cellular redox balance. It is known that ROS can function as a second messenger to activate NF- κ B, which orchestrates the expression of a spectrum of genes involved in the inflammatory response.

Overproduction of NO contributes to the pathogenesis of chronic arthritis. In humans, increased circulating levels of nitrate/nitrite are present in arthritic patients and the synovial tissues of patients with RA express iNOS (Sakurai et al., 1995) and produce abnormally high amounts of NO (Connor et al., 1995).

Ozone therapy regulated NO generation so PG/PS+Ozone did not differ from Control (-). In contrast, an overproduction of NO in PG/PS and PG/PS+Oxygen groups was observed (Fig. 4B). Ozone's regulatory effects on iNOS have been demonstrated in ischemia/reperfusion injury (Ajamiéh et al., 2004; Leon Fernandez et al., 2008).

The inflamed joint in RA is the predominant source of NO (Pham et al., 2003). Several investigators found correlations between serum nitrite concentration and RA disease activity or radiological progression while others did not find such correlations (Gonzalez-Gay et al., 2004). Several different cell types are capable of generating NO in the inflamed synovium, including osteoblasts, osteoclasts, macrophages, fibroblasts, neutrophils and endothelial (Nagy et al., 2007). NOS inhibition was reported to decrease disease activity in experimental RA (van't Hof et al., 2000). Recently, it has been demonstrated that T cells from RA patients produce more than 2.5 times NO than healthy donor T cells (Nagy et al., 2008). Ozone protective effects against NO overproduction are likely a consequence of ozone's actions on NF- κ B since iNOS is regulated at the transcriptional level by this nuclear factor.

Besides NO other redox markers were studied (Table 1). PG/PS and PG/PS+Oxygen exhibited a low activity of SOD while PG/PS+Ozone and Control (-) did not show significant differences. Increase of NO and decrease of SOD represents a pathological "explosive mixture". Accumulation of superoxide radicals in the presence of overproduction of NO leads to peroxynitrite formation a well-known cytotoxin. Modestly increasing superoxide and NO each at a 10-fold greater rate will increase peroxynitrite formation by 100-fold. Under pro-inflammatory conditions, as it happens in RA, simultaneous production of superoxide and NO can be strongly activated to increase production 1000-fold, which will increase the formation of peroxynitrite by a 1,000,000-fold (Pacher et al., 2007).

SOD mimetics also reduced nitrotyrosine formation and joint inflammation in a rat model of collagen-induced arthritis (Salvemini et al., 2001). There is additional evidence of protein nitration in joints from rheumatoid patients, who disclose increased levels of nitrotyrosine in plasma and synovial fluid, and nitrotyrosine formation localized within macrophages and vascular smooth muscle in the inflamed synovium (Sandhu et al., 2003).

NO, superoxide, and peroxynitrite generation represents delayed phenomena in the immunologic and inflammatory series of events associated with arthritis (Mabley et al., 2002).

Table 1

Superoxide Dismutase (SOD), Catalase (CAT) activities, Fructolysine content and Redox Index (CAT/SOD) in rat spleen homogenates in PG/PS-induced arthritis treated with ozone/oxygen.

GROUPS	SOD (U/mg protein)	CAT (U/mg protein)	FRUCTOLYSINE (relative content/mg protein $\times 10^3$)	CAT/SOD Redox Index
Control (–)	25.87 \pm 0.85 ^a	212.2 \pm 13.8 ^a	2.73 \pm 1.63 ^a	8.18 \pm 0.95 ^a
PG/PS	2.73 \pm 0 ^b	182.1 \pm 0 ^a	12.33 \pm 4.33 ^b	66.7 \pm 0 ^b
PG/PS+Ozone	34.99 \pm 0.8 ^a	391.07 \pm 2.6 ^b	1.8 \pm 0.3 ^a	11.18 \pm 0.53 ^c
PG/PS+Oxygen	11.49 \pm 1.28 ^c	207.9 \pm 19.6 ^a	9.53 \pm 2.15 ^b	18.12 \pm 2.38 ^d

Control (–) (without treatment), Peptidoglycan–Polysaccharide (PG–PS), PG/PS+Ozone (PG/PS+O₃) and PG/PS+Oxygen (PG/PS+O₂) groups. Data represent the mean of three different independent experiments. Means having different letters indicate significant differences ($P < 0.05$) between groups.

Increase of SOD promotes hydrogen peroxide formation which may produce additional damage to biomolecules. Nevertheless, hydrogen peroxide is reduced if catalase increases its activity. PG/PS+Ozone showed an increment in comparison with all other experimental groups (Control, PG/PS and PG/PS+Oxygen) (Table 1). Control (–) does not need the increase of CAT but PG/PS and PG/PS+Oxygen are in severe oxidative stress and their catalase activity is low; in consequence they displayed damage. This result is in line with CAT/SOD ratio which is a Redox Index that indicates the redox metabolic balance. Although PG/PS+Ozone did not achieve Control (–) levels there was a decrease of this index in comparison with PG/PS and PG/PS+Oxygen groups (Table 1) which indicates an improvement of the redox balance by ozone treatment.

Fructolysine is an indicator of protein glycation due to the fact that it is a precursor of glycation end-products (AGE). When there is formation of these glycated proteins ROS are generated. It has been suggested that several self-proteins are able to be disease-driving autoantigens in RA. Between them are post-translational modifications such as glycosylation (Alivernini et al., 2008). Ozone treatment maintained fructolysine concentrations similar to Control (–) and it may contribute to reduce the erosive immune response. In contrast, PG/PS and PG/PS+Oxygen increased fructolysine levels with regard to Control (–) and PG/PS+Ozone.

RA is an extremely complex disease, with an etiology involving the interplay of (at least) genetic predisposition, environmental factors, and immunity to self-molecules made immunogenic by posttranslational modification (Klareskog et al., 2006).

A new hypothesis of autoimmunity, currently gaining experimental support, proposes to look for an opposite causality; rather than RA causing increased glycation, perhaps individuals prone to non-enzymatic glycation of proteins (for whatever reason) may be more likely to develop RA since glycation forms new antigenic determinants on proteins. This type of connection has been already demonstrated for a different class of posttranslational protein modification, represented by conversion of arginine to citrulline, in a large subset of RA patients (van Venrooij and Pruijn, 2000). This finding is likely connected with overproduction of NO found in patients and animal models of RA since arginine is the substrate of nitric oxide synthase (NOS) which produces L-citrulline and NO.

There are many examples of different posttranslational modifications within target proteins leading to the breaking of immunological tolerance and induction of autoimmunity (Atassi and Casali, 2008; Doyle and Mamula, 2005). The process would ultimately lead to the development of “genuine” autoantibodies by epitope spreading. Abnormal glycation of IgG in patients with RA was already related to induction of autoantibodies and it is presumed the existence of a link between AGE formation and the induction of autoimmunity (Vytasek et al., 2010).

On the other hand, RA is the disease with the highest mean level of pentosidine (AGE) in serum/plasma of all diseases investigated so far, even higher than the mean level found in sera/

plasma of diabetic patients (Miyata et al., 1998). Metotrexate and the monoclonal antibody Infliximab reduced levels of pentosidine in RA. These findings underline the role of AGE in RA.

In summary, ozone treatment administered by intra-articular way in PG/PS-induced arthritis improved the inflammatory process. Pleiotropic ozone's effects clarify its therapeutic efficacy in RA. In spleen homogenates ozone treatment decreased the levels of TNF- α and IL-1 β transcripts and TNF- α concentration whereas NO concentration was reestablished after ozone oxidative post-conditioning. Besides a reduction of oxidative stress and the reestablish of fructolysine (AGE precursor) levels were achieved. All these molecules are goals of different drugs and all of them are targets of ozone therapy. Other studies are in progress such as ozone's protective effects in other experimental models as well as in controlled clinical trials. Besides other ozone's administration routes must be studied since RA is considered as a systemic autoimmune disease.

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