



## Immunopharmacology and inflammation

# Medical ozone increases methotrexate clinical response and improves cellular redox balance in patients with rheumatoid arthritis



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## ABSTRACT

Medical ozone reduced inflammation, IL-1 $\beta$ , TNF- $\alpha$  mRNA levels and oxidative stress in PG/PS-induced arthritis in rats. The aim of this study was to investigate the medical ozone effects in patients with rheumatoid arthritis treated with methotrexate and methotrexate+ozone, and to compare between them. A randomized clinical study with 60 patients was performed, who were divided into two groups: one (n=30) treated with methotrexate (MTX), folic acid and Ibuprofen (MTX group) and the second group (n=30) received the same as the MTX group+medical ozone by rectal insufflation of the gas (MTX+ozone group). The clinical response of the patients was evaluated by comparing Disease Activity Score 28 (DAS<sub>28</sub>), Health Assessment Questionnaire Disability Index (HAQ-DI), Anti-Cyclic Citrullinated (Anti-CCP) levels, reactants of acute phase and biochemical markers of oxidative stress before and after 20 days of treatment. MTX+ozone reduced the activity of the disease while MTX merely showed a tendency to decrease the variables. Reactants of acute phase displayed a similar picture. MTX+ozone reduced Anti-CCP levels as well as increased antioxidant system, and decreased oxidative damage whereas MTX did not change. Glutathione correlated with all clinical variables just after MTX+ozone.

MTX+ozone increased the MTX clinical response in patients with rheumatoid arthritis. No side effects were observed. These results suggest that ozone can increase the efficacy of MTX probably because both share common therapeutic targets. Medical ozone treatment is capable of being a complementary therapy in the treatment of rheumatoid arthritis.

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## 1. Introduction

Rheumatoid arthritis (RA) is the commonest inflammatory joint disease, afflicting about 1% of the world population.

The ultimate therapeutic goal in RA treatment is remission or at least low disease activity, which may not always be achieved with methotrexate monotherapy, so that combination therapy appears to be better. Recent reports found that most dissatisfied arthritis patients are likely to seek the option of complementary and alternative medicine (Efthimiou et al., 2010).

On the other hand, the combination of carnosine (antioxidant) with methotrexate decreased inflammation in rheumatoid arthritis model in rats more effectively than treatment with

methotrexate alone. This result is in compliance with other studies on combination of naturally occurring substances and methotrexate, where the combination proved more effective than monotherapy (Bauerova et al., 2010). A combination of MTX with drugs that decrease inflammation in RA and also show antioxidant properties would be better than MTX monotherapy.

MTX remains the first line drug for the treatment of RA. Although some mechanisms have been proposed, most of the available evidence supports the adenosine theory which plays a role in MTX efficacy in RA (Roopjet et al., 2014). Adenosine interacts with specific cell surface receptors, with subsequent inhibition of inflammation mediators (Cronstein, 2005).

The Reactive Oxygen Species (ROS) are another factor involved in RA. A great amount of ROS produced in arthritic joints (Lisa et al., 2012) has contributed to the fact that many studies associate oxidative stress with RA. Indeed, oxidative stress is closely related

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with RA and the maintenance of a proper redox balance seems to be a critical step in order to improve the clinical outcome.

At present, there is no available direct cure for RA; the main goals of the treatment are therefore to ameliorate the symptoms of the disease (i.e., diminish pain and decrease inflammation and joint destruction). In addition, the majority of these drugs have numerous side effects (Kyra et al., 2007).

Medical ozone is an ozone/oxygen mixture administered at low concentrations. It is able to reestablish cellular redox balance and increase adenosine availability through an ozone oxidative pre-/postconditioning mechanism (León et al., 1998). The proposed mechanism has been validated in pathological conditions such as ischemic syndrome, diabetes and diabetic foot, disc hernia, pain and other diseases (León, 2014).

Adenosine and redox status are common therapeutic targets of MTX and medical ozone so it is possible to hope that the combination of both therapeutic concepts could increase the MTX clinical response. Previous to this work ozone protective effects on PG/PS- induced arthritis in rats on inflammation and reduction of IL-1 $\beta$  and TNF- $\alpha$  mRNA levels were demonstrated (Jaqueline et al., 2013).

Taking into account that medical ozone shares therapeutic targets with MTX, the aim of this project was to study the effects of medical ozone in patients with RA being treated with methotrexate and methotrexate+ozone (rectal insufflations), and to compare both groups with regard to MTX clinical response as well as to investigate whether medical ozone antioxidant properties are associated with the clinical outcome.

## 2. Materials and methods

### 2.1. Study design

This randomized controlled clinical study was approved by the joint institutional review board (Scientific and Ethics Committees from the National Institute of Rheumatology, Ministry of Public Health, Cuba, and Pharmacy and Food Institute, University of Havana, Cuba) in accordance with the principles of the Declaration of Helsinki (2005). All patients gave their informed consent to enrollment after receiving adequate information concerning the study (characteristics of the study, benefits and possible side effects). Before enrollment, all participants attended a training program to familiarize them with the study objectives and treatment plans. The personnel involved emphasized that all participating physicians would treat each patient according to the randomized scheme of treatment through a Research Randomizer Form.

To calculate the size of the sample, the Medstat Systems, Inc. (version 2.1, 1989; Fridley, MN, USA) method was used. The statistical difference between the beginning and the end of ozone therapy was 0.2 with a type 1 error of 0.05 (Levy and Lemeshow, 1991). The target level of enrollment was determined at 27 patients. Assuming that 10% of the patients studied would be lost to follow-up, 30 patients were included.

Inclusion criteria: Adult patients (> 18 years) of both sexes and different ethnic origins with a diagnosis of RA who fulfilled the revised American Rheumatism Association's (Arnett et al., 1988) criteria for RA (morning stiffness, swelling of hand joints, swelling of three or more joints, symmetric swelling of joints) were eligible to participate in the study. Patients of the National Institute of Rheumatology, Ministry of Public Health, Cuba who accomplished the following criteria were chosen: Disease Activity Score 28 (DAS<sub>28</sub> > 3.2  $y$   $\leq$  5.1) whose examination was carried out under blinded conditions by a physician different to the one who selected the patients according to a randomized scheme of treatment and a preliminary brief medical history. The Health Assessment

Questionnaire-Disability Index: (HAQ-DI, according to the validated Spanish version) (Cardiel et al., 1993), "C" Reactive Protein (CRP > 6 mg/l in serum), Erythrocyte Sedimentation Rate (ESR > 8 mm for males and 16 mm for females) and anti-Cyclic Citrullinate Peptides (anti-CCP > 10 U/ml in serum) as well as patients with disease duration longer than five year were included. The exclusion criteria were: patients with any history of chronic conditions such as liver disease, diabetes mellitus, respiratory disorders, cardiovascular diseases and alcohol usage and smoking were not included in the study. Patients with overlapping syndrome, cancer, or other associated autoimmune disorders or who were pregnant were also excluded. Those patients who had been receiving corticosteroid agents and were under treatment with disease modifying anti-rheumatic drugs and anti-TNF or other biological agents for at least 3 months before the study date were also excluded.

The patients were randomized into two different groups of treatment: (MTX group), MTX 12.5 mg, intramuscular (i.m.), once/week (every Monday from 9:00–10:00 in the morning)+Ibuprophen (400 mg, oral), one Tablet each 8 h+Folic Acid (5 mg, oral), one Tablet/day from Wednesday to Saturday. (MTX+ozone group), same MTX group+medical ozone which was generated by an OZOMED unit, Cuba. 20 treatments by rectal insufflations (five/week from Monday to Friday). 25 mg/l to 40 mg/l of ozone in stepped application and in increasing order were administered as follows:

1st week: 25 mg/l, 100 ml; 2nd week: 30 mg/l, 150 ml; 3rd week: 35 mg/l, 200 ml; 4th week: 40 mg/l, 200 ml.

Medical personnel were instructed to report all adverse reactions, whether described in the package circulars of the study medications or not.

#### 2.1.1. Evaluation of disease activity

Changes in the evolution of disease, that is, clinical improvements through suitable indices of activity (clinical parameters) as well as anti-CCP antibodies and redox status determinations before the beginning and at the end of clinical study (21 days) were assessed. Each patient was his/her own control (i.e. before medical ozone treatment).

The main variables considered were:

Clinical parameters: DAS<sub>28</sub>  $\leq$  3.2 (Prevo et al., 1995), decrease of HAQ-DI and a reduction of pain intensity (VAS  $\geq$  50%). A visual analogical scale (VAS) from '10' to '100' was evaluated. This was classified as '10' (minimum pain intensity) and '100' (maximum pain intensity). No pain was considered as "0" as well as a decrease in the reactants of acute phase and anti-CCP.

Secondary variables considered were: (a) Serum levels of injury markers such as advanced oxidation protein products (AOPP), nitric oxide (NO), total hydroperoxides (TH) and malondialdehyde (MDA). (b) Serum levels of protective redox markers such as reduced glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD) activities. c) Side effects.

A result was considered good when MTX+ozone group decreased ( $P < 0.05$ ) with regard to MTX group at the end of the study (21 days) in: DAS<sub>28</sub>, HAQ-DI, pain intensity, acute phase reactants and auto antibody anti-CCP levels. An increase in endogenous antioxidants (GSH, SOD and CAT) and a decrease in injury redox markers (NO, AOPP, TH and MDA) were also considered to be good results.

The therapeutic response was considered successful if 70% of the patients treated with MTX+ozone had a positive outcome, taking into account the main variables, and if this improvement was 30% higher than that in the patients treated with MTX by itself.

## 2.2. Biochemical determinations

Blood samples for biochemical analysis were obtained after a 12-h overnight fast, at the beginning, and 24 h after the last MTX+ozone and MTX treatments.

Anti-CCP antibodies were determined using an ELISA kit (DRG, DRG Diagnostics, GmbH, Germany) (sensitivity 90%, specificity 98.3% and diagnostic efficacy 95.3%). "C" Reactive Protein was determined with the test for agglutination (Latex CRP test) (BioSystems S.A. Reagent and Instruments Costa Brava 30, Barcelona, Spain), and the Erythrocyte sedimentation rate (ESR) was obtained using Westergren's quantitative method.

Redox parameters were determined by spectrophotometric methods using an BOECO Model S 220 Spectrophotometer, Germany.

Superoxide dismutase (SOD) activity was measured using kits supplied by Randox Laboratories Ltd., Ireland (Cat. no. SD125 and no. RS505). Catalase (CAT) activity was measured by following the decomposition of hydrogen peroxide at 240 nm at 10 s intervals for 1-min (Boehringer\_Mannheim, 1987). After precipitation of thiol proteins using trichloroacetic acid 10%, reduced glutathione (GSH) was measured according to the method of Sedlak and Lindsay (1968) with Ellman's reagent [5'5 dithiobis (2-nitrobenzoic acid) $10^{-2}$  M (Sigma St. Louis, MO, USA)]; absorbance was measured at 412 nm.

Nitrite/nitrate levels as a measure of nitric oxide (NO) were determined by the Griess reaction after first converting nitrates to nitrites using nitrate reductase (Boehringer- Mannheim Italy SpA, Milan, Italy) (Granger et al., 1995). The advanced oxidation protein products (AOPP) were measured as the oxidation of iodide anion to diatomic iodine by advanced oxidation protein products (Witko-Sarsat et al., 1998). Quantification of total hydroperoxides (TH) was measured by Bioxytech H2O2-560 kit (Oxis International Inc., Portland, OR, USA). Concentrations of malondialdehyde (MDA) were analyzed using the LPO-586 kit obtained from Calbiochem (La Jolla, CA).

## 2.3. Statistical analysis

The OUTLIERS preliminary test for detection of error values was initially applied. Afterward, data were analyzed by one way analysis of variance (ANOVA) followed by a homogeneity variance test (Bartlett-Box). In addition, a multiple comparison test was used (Student-Newman-Keuls test). Student *t*-test for independent samples, canonical discriminate analysis and *t*-test of paired samples were applied. Pearson's lineal correlations were used. Results are presented as means  $\pm$  standard error of the mean (S.E.M.). The level of statistical significance used was at least  $P < 0.05$ .

## 3. Results

### 3.1. General characteristics of the patients involved in the study

In relation to the baseline characteristics (Table 1), both groups were similar at randomization ( $P > 0.05$ ). Women were the predominant sex. Differences between groups (MTX and MTX+ozone) with regard to age, previous therapy and evolution time of RA were not observed. Results in the context of sex were similar to other reports. Women are affected by RA about three times more often when compared with men, but the cause of this difference is unknown. An increase in Caucasian patients in both groups was observed.

### 3.2. Picture of main variables at the beginning and at the end of the study

Before the beginning of the study, the group treated with

**Table 1**

Clinical picture of patients with rheumatoid arthritis.

Demographic data/patient histories	MTX Group	MTX+Ozone Group
	(n=30)	(n=30)
Women (n%)	27/90%	28/93%
Men (n%)	3/10%	2/7%
Age (years)	53 $\pm$ 8 <sup>(a)</sup>	57 $\pm$ 7 <sup>(a)</sup>
Previous therapy		
(Methotrexate) (n%)	30/100%	30/100
Corticosteroids	0	0
Evolution time of the disease (years)	7 $\pm$ 2 <sup>(a)</sup>	11 $\pm$ 3 <sup>(a)</sup>
Race		
Caucasian	17/56%	21/71%
Non-Caucasian	13/44%	9/29%

MTX group: Methotrexate+Ibuprophen+folic acid. MTX+ozone group: Same group MTX+medical ozone.

The data reflecting age and progress through time of the disease are: mean  $\pm$  S.E.M. of each group. Mean values with different letters indicate significant differences ( $P < 0.05$ ) between both groups. (a)  $P$  minor 0.05.

Statistical Tests: One-way Analysis of Variance (ANOVA) followed by Student-Newman-Keuls test.

MTX+medical ozone showed a higher pain intensity than MTX group. At the end of the experiment (21 days), MTX+medical ozone showed a decrease in pain intensity while there was no modification in pain intensity in the MTX group and a statistical differences ( $P < 0.05$ ) between both groups at the end of the clinical study was observed (Table 2).

There were no differences between DAS<sub>28</sub> and HAQ-DI at the beginning of the study (Table 2). The DAS<sub>28</sub> was decreased in the MTX+medical ozone group, while there was no change in the MTX group. At the end of the clinical study, differences between both groups were observed. HAQ-DI showed similar picture to DAS<sub>28</sub>. MTX+medical ozone improved patients' disabilities, whereas the MTX group terminated without change. Again, differences between both groups at the end of the study were found. Both reactants of the acute phase (CRP and ESR) were decreased in the MTX+ozone whereas the patients in the MTX group showed no change at the end of the study. This shows that MTX+medical ozone were able to reduce the inflammatory response in patients with RA.

**Table 2**

Clinical characteristics of patients with RA at the beginning and at the end of the study.

Clinical variables	MTX Group (n=30)		MTX+Ozone Group (n=30)	
	Start	End	Start	End
Pain	8.2 $\pm$ 0.4 <sup>(a)</sup>	7 $\pm$ 0.6 <sup>(a)</sup>	9.2 $\pm$ 0.3 <sup>(a)</sup>	4.7 $\pm$ 0.3 <sup>(b,c)</sup>
DAS <sub>28</sub>	5.6 $\pm$ 0.3 <sup>(a)</sup>	5.2 $\pm$ 0.3 <sup>(a)</sup>	6.4 $\pm$ 0.2 <sup>(a)</sup>	3.2 $\pm$ 0.3 <sup>(b,c)</sup>
HAQ-DI	1.3 $\pm$ 0.1 <sup>(a)</sup>	1.1 $\pm$ 0.1 <sup>(a)</sup>	1.8 $\pm$ 0.1 <sup>(a)</sup>	0.7 $\pm$ 0.0 <sup>(b,c)</sup>
CRP (mg/l)	21 $\pm$ 7 <sup>(a)</sup>	13 $\pm$ 4 <sup>(a)</sup>	16 $\pm$ 4 <sup>(a)</sup>	5 $\pm$ 1 <sup>(b,c)</sup>
ESR	40 $\pm$ 6 <sup>(a)</sup>	40 $\pm$ 6 <sup>(a)</sup>	36 $\pm$ 6 <sup>(a)</sup>	20 $\pm$ 4 <sup>(b,c)</sup>
Anti-CCP (U/l)	102 $\pm$ 3 <sup>(a)</sup>	119 $\pm$ 3 <sup>(b)</sup>	107 $\pm$ 6 <sup>(a)</sup>	89 $\pm$ 3 <sup>(b,c)</sup>

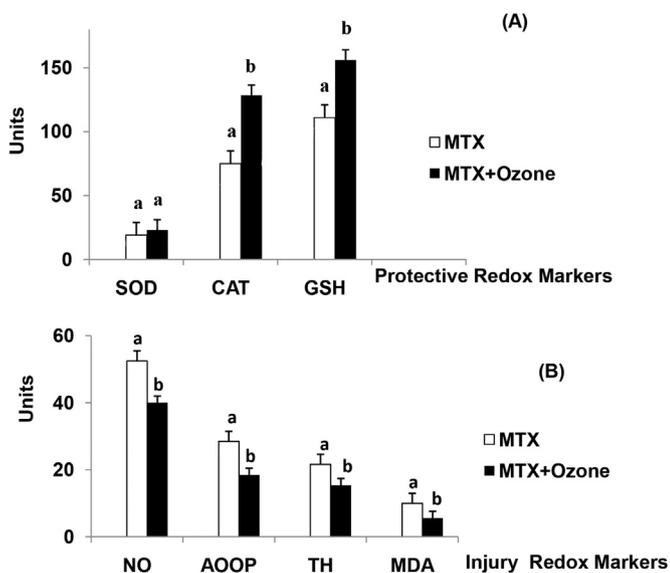
MTX group: Methotrexate+Ibuprophen+folic acid. MTX+ozone group: same MTX Group+medical ozone.

DAS<sub>28</sub>, Disease Activity Score 28 (low activity  $\leq 3.2$ ; moderate activity  $> 3.2$  y  $\leq 5.1$ ; high activity  $> 5.1$ ); HAQ-DI, Health Assessment Questionnaire-Disability Index ( $+ > 1.25$ ); CRP, "C" Reactive Protein ( $+ > 6$  mg/l in serum); ESR, Erythrocyte Sedimentation Rate (men 7–8 mm, women 11–16 mm); Anti-CCP, Anti-cyclic citrullinate peptides ( $\geq 10$  U/ml in serum).

"Start" means before the beginning of the study and "End" after the final ozone treatment (21 days after starting of the study).

Statistical tests: student *t*-test for independent samples, canonical discriminate analysis and *t*-test of paired samples were applied.

Data represent the mean  $\pm$  S.E.M. Mean values with different letters indicate significant differences ( $P < 0.05$ ). (c)  $P < 0.05$  21 days (at the end of the study) "MTX+ozone group" vs "MTX Group".



**Fig. 1.** The redox status of patients with RA in MTX and MTX+ozone groups at the end of the study. (A) Protective redox markers, (B) Injury redox markers. The units of each marker are: SOD (U/ml/min) and CAT (U/l/min) activities, GSH ( $\mu\text{M}$ ), NO ( $\mu\text{M}$ ), AOPP ( $\mu\text{M}$ ), TH ( $\mu\text{M}$ ), MDA ( $\mu\text{M}$ ). Data represent the mean  $\pm$  S.E.M. of each group. Data analysis for each group was made by *t*-test. (\*)  $P < 0.05$  MTX vs MTX+ozone.

Anti-cyclic citrullinate peptides (anti-CCP) levels in patients with RA are shown in Table 2. Medical ozone reduced autoantibodies against cyclic citrullinated peptides (CCP): by contrast, they were increased in patients without ozone treatment. In the same way, autoantibody levels in the MTX+medical ozone group were lower than in the MTX group at the end of study, so that these results indicate that medical ozone is able to achieve a reduction of autoimmune response and they are in line with clinical evaluations (DAS<sub>28</sub>, HAQ-DI and reactant of acute phase).

### 3.2.1. Redox biomarker levels in both groups at the end of the study

Plasmatic determinations of protective (antioxidants) and injury (pro-oxidants) redox markers in both groups of patients were studied (Fig. 1).

MTX+medical ozone increased the capacity of the antioxidant endogenous system to resist oxidative injury, producing as a result a decrease in the damage to biomolecules (lipids and proteins) as well as in TH levels and nitric oxide concentrations. By contrast, patients who received no ozone showed a reduction in antioxidant defense and a higher level of damage (Fig. 1A and B).

In order to clarify whether there was any relationship between the redox markers and the clinical outcome, the correlations between the variables were evaluated. Only after MTX+medical ozone could correlations be found. GSH was the only protective redox marker that correlated with all clinical variables (GSH vs CRP=0.68, ESR=0.63, DAS<sub>28</sub>=0.57 and HAQ-DI=0.72) while there was SOD activity in some of them (SOD vs CRP=0.5, ESR=0.51 and HAQ-DI=0.61).

## 4. Discussion

Medical ozone increased the therapeutic effectiveness of MTX in patients with RA. The improvement of clinical status (pain reduction, DAS<sub>28</sub>, HAQ-DI and reactant of acute phase) as well as the reduction of immune response (anti-CCP) and oxidative stress are therefore demonstrated. These effects seem to be a result of the fact that both agents share common therapeutic targets, such as redox balance and adenosine actions.

Proteolytic activity is not the only source of cartilage and bone destruction associated with RA. ROS also have direct effects as they oxidize and degrade the major components of cartilage and bone, including collagen and hyaluronic acid (HA) (Hadjigogos, 2003). The perpetuation of the inflammatory process is considered to be mediated by a number of cytokines which stimulate the oxidase NADPH activation, thus producing superoxide radicals (Moynagh, 2005).

SOD (superoxide dismutase) is part of the antioxidant endogenous system and it is a scavenger of superoxide radicals. This system enhances the otherwise slow spontaneous breakdown of superoxide radicals, forming the less toxic hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Superoxide radicals inhibit the synthesis of matrix components including proteoglycans via chondrocytes. Oxygen radicals can cause low levels of collagen fragmentation and enhance collagen fibril cross-linking (Carol and Hani, 2004). Correlations between SOD and clinical variables suggest that the ozone that stimulates SOD activity (Gregorio et al., 2005) is also able to capture superoxide radicals in order to avoid joint injury.

CAT activity protects the cells from the accumulation of hydrogen peroxide. Decreases in catalase activity in rheumatoid arthritis have been observed (Krishna and Chandrasada Gopan, 2008). Medical ozone increased catalase activity and reduced hydrogen peroxide levels, as well as preventing damage to proteins and lipids.

It has been suggested that, in the process of degradation of joint cartilage, active forms of oxygen are involved. The MTX+medical ozone combination caused a decrease in Advanced Oxidation Protein Products (AOPPs) in patients with RA. Reduction of oxidative stress by ozone not only decreased ROS concentrations, which are otherwise able to cause degeneration in joint cartilage, but also reduced metalloproteinase activities (Filippin et al., 2008), thus preventing their participation in a degeneration of the extracellular matrix.

On the other hand, lipid peroxidation has been implicated in the pathogenesis of inflammatory arthritis. During lipid peroxidation, polyunsaturated fatty acids are oxidized to produce lipid peroxyl radicals that in turn lead to further oxidation of polyunsaturated fatty acid in a perpetuating chain reaction that can lead to cell membrane damage. Matrix degradation arising from cytokine-stimulated chondrocytes was shown to be primarily due to lipid peroxidation (Carol and Hani, 2004). MTX+medical ozone reduced MDA concentrations indicating a reduction in lipid peroxidation, and it suggests a decrease in matrix injury.

Several studies in patients with RA have documented evidence for increased endogenous NO synthesis, suggesting that an overproduction of NO may be important in the pathogenesis of RA. The inflamed joint in RA is the predominant source of NO. Modification of amino acids by nitrosylation and nitration can alter protein structure and impair biological function, leading to cell death. MTX+medical ozone had reduced NO concentrations at the end of the clinical study. It suggests a regulation of NO which promotes a reduction of amino acids oxidative modification as well as apoptosis.

Moreover, glutathione is considered the most important hydro-soluble non-enzymatic anti-oxidant, as it participates in numerous ox-reduction reactions. GSH levels play a role in RA, though GSH concentrations may have an additional meaning in RA patients treated with MTX+medical ozone in our experimental conditions. This tripeptide was the only redox marker correlating with all clinical variables immediately after MTX+ozone treatment, which suggests it is involved in the effectiveness of MTX+medical ozone. Besides, it is closely associated with ozone metabolites that have pharmacological properties. It has been suggested that membrane-associated ozone peroxides could act as second messengers via cysteine residues and/or reduction through

GSH in a less aggressive way than the superoxide radicals  $\bullet\text{O-O-}$  and  $\text{H}_2\text{O}_2$ , and take over regulation of the anti-oxidants, i.e., without SOD and catalase demand as in the oxidative stress processes of relevant pathological conditions (Renate et al., 2012).

GSH in one of two major sulfur-based antioxidant systems and acts as a radical scavenger by trapping ROS that would otherwise react with cellular thiols and disturb the redox equilibrium. Despite the oxidizing environment, disulfide bonds in the extracellular domains of some cell surface proteins can be cleaved and exist as a thiol group. The best-characterized examples of proteins containing  $-\text{SH}$  groups on the cell surface are  $\text{CD4}^+$  T cells which are critical for the development of arthritis. Clearly, regulation of the numbers of thiol groups on the T cell membrane is one of the key mechanisms regulating T cell activity in vivo and it thereby also determines the development of autoimmune disease such as arthritis (Gelderman et al., 2006). Increase of GSH concentrations and its correlations with clinical variables after  $\text{MTX} + \text{ozone}$  suggest that this drug combination contributes not only to improve cellular redox balance, but also to regulate essential thiol groups and  $\text{CD4}^+$  T cell activity in patients treated with  $\text{MTX} + \text{medical ozone}$ .

$\text{MTX} + \text{medical ozone}$  decreased redox injury markers in patients with RA. These results may indicate that cartilage and bone injury were diminished. It is in line with the anti-CCP antibody levels that decreased after  $\text{MTX} + \text{medical ozone}$  application. Anti-CCP antibodies are actively produced or enriched at the site of inflammation (joints and synovial tissue) and may play an active role in the pathogenesis of anti-CCP positive RA by enhancing oxidative stress in rheumatoid joint (Snir et al., 2010). Indeed, high titers of anti-CCP antibodies have been associated with an erosive disease course and outcome in RA (Silveira et al., 2007).

The results of clinical and cellular redox balance in patients treated with  $\text{MTX} + \text{medical ozone}$  suggest that ozone increases the therapeutic efficacy of  $\text{MTX}$  in RA.  $\text{MTX}$  and medical ozone share common pharmacological mechanisms: For one thing,  $\text{MTX}$  promotes constitutive ROS production by phagocytes in response to antigens affecting T cell–antigen interactions, and it possibly induces apoptosis of autoreactive arthritogenic T cells, thereby inhibiting autoimmune responses (Carol and Hani, 2004). On the other hand, medical ozone regulates ROS production, achieving a cellular redox balance through stimulation of the endogenous antioxidant defense system. The result is a decrease in cellular and tissue injury (León, 2014).

Another therapeutic target of both agents is adenosine: linkage of  $\text{MTX}$  with adenosine and its binding to  $\text{A}_2$  and  $\text{A}_3$  adenosine receptors result in a favorable situation which is probably one of the most important anti-inflammatory mechanisms of  $\text{MTX}$  (Roopjet et al., 2014). Besides, medical ozone promotes adenosine accumulation in ischemic conditions because it blocks adenosine degradation to hypoxanthine and xanthine (Peralta et al., 2000). In this way, accumulated adenosine could activate adenosine receptors thus resulting in an anti-inflammatory effect. This means that anti-inflammatory actions are strengthened when both therapeutic agents act together.

To summarize, under our experimental conditions, the combination of  $\text{MTX} + \text{medical ozone}$  improved the clinical status of patients suffering from RA when compared with patients treated using  $\text{MTX}$  by itself. There was a correspondence between clinical variables, redox cellular balance indices and anti-CCP antibody levels. Taken together, the results suggest that medical ozone can act as an agent that increases the clinical efficacy of  $\text{MTX}$  in RA. The patients who were included in the study are being monitored on a post-study basis once a month for clinical controls and every six months for redox controls. In order to study patients resisting  $\text{MTX}$  application as well as to assess the autohemotherapy ozone administration route in RA patients, other clinical studies are in progress.

## Conflict of Interest

The authors declare that they have no conflict of interest.

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