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Medical Ozone Effects and Innate Immune Memory in Rheumatoid Arthritis Patients Treated with Methotrexate+Ozone After a Second Cycle of Ozone Exposure

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Abstract

Combined methotrexate+medical ozone therapy administered by rectal insufflation has demonstrated an increase in clinical efficacy and a reduction of the hepatotoxicity risk in Rheumatoid Arthritis (RA) patients. Although more than one ozone cycle is a routine procedure in clinical practice ensuring efficacy and safety, no study investigating the clinical and redox characterization at the end of two or more cycles of ozone treatment in RA patients has yet been performed. The aim of this study was to assess the beneficial effects of a second ozone cycle using clinical indicators and redox biomarkers, and its relationship with innate immune memory after exposure to the second ozone cycle. In RA patients treated with Methotrexate+ozone, a second ozone exposure promoted powerful clinical and redox responses. An increased reduction of inflammation, disability and a decrease in Anti-Cyclic Citrullinate Peptides (Anti-CCP) levels were observed. The impact on cellular redox status was greater than the clinical responses. All redox biomarkers (6/7) returned to a normal interval. A marked reduction of those mediators contributing to cartilage and bone injury was observed. The concentrations of γ-glutamyl transferase (an inducer of osteoclastogenesis and glutathione depletion) corresponded with the decrease in Anti-CCP and the increase in reduced glutathione.

RA patients treated with Methotrexate+ozone showed increased clinical and redox responses after a second cycle of ozone exposure. This potentization seems to be mediated by innate immune memory due to the close relationship between the proposal targets involving an innate immune memory and those that are regulated by medical ozone.

Keywords: Ozone; Rheumatoid arthritis; Innate immune memory

Introduction

Medical ozone is an ozone/oxygen mixture which has demonstrated its clinical efficacy in various diseases such as ischemic syndrome, diabetes and neuro infectious foot, disc hernia, pain and other diseases which have chronic oxidative stress as common pathological characteristic. Ozone oxidative pre/post conditioning is a mechanism that stimulates the endogenous antioxidant defence system consequently regulating a cellular redox balance which became disrupted in the above mentioned disorders [1,2].

Rheumatoid Arthritis (RA) is classified as an autoimmune disease. Reactive Oxygen Species (ROS) are produced to a great extent in arthritic joints [3], so that many studies have associated

oxidative stress with RA. Most of these papers have studied the role of ROS overproduction in cartilage and bone injury. Oxidative stress is in fact closely related to RA, and the maintenance of a proper redox balance seems to be a critical step in order to improve the clinical outcome.

Methotrexate (MTX) + medical ozone administered by rectal insufflation is a combined therapy that has increased the anti-inflammatory response and decreased the hepatotoxicity risk as compared with MTX-treated patients [4,5].

When ozone rectal insufflation is administered, the therapeutic schedule generally covers more than one cycle of ozone treatment (each cycle consisting of 20 treatments). The second cycle is able to increase or to maintain an improvement in clinical response to ozone. The cycles are separated by intervals of three months. This is a routine procedure in clinical practice to provide the efficacy and safety that have been demonstrated on a scientific basis [6]. However, no study investigating the clinical and redox characterization at the end of a second or several cycles of ozone treatment in RA patients has yet been performed.

Taking into account the good clinical response achieved in RA patients treated with more than one cycle of MTX+ozone, the aim of this study was to assess the beneficial effects of a second ozone cycle when compared with results from the first cycle using clinical indicators and redox biomarkers and its relationship with innate immune memory at the end of the second ozone cycle in RA patients treated with the MTX+ozone combined therapy.

Materials and Methods

Study design

This controlled clinical study was approved by the joint institutional review board (Scientific and Ethics Committees of 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 enrolment after receiving adequate information concerning the study (characteristics, benefits and possible side effects). Before enrolment, all participants attended a training program to familiarize them with the study objectives and treatment plans.

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 end of ozone therapy was 0.2 with a type 1 error of 0.05 [7]. The target level of enrolment was determined in 20 patients.

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 [8] 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 28 >3.2 y≤5.1) The Health Assessment Questionnaire-Disability Index: (HAQ-DI, according to the validated Spanish version [9], and anti-Cyclic Citrullinate Peptides (anti-CCP >10 U/ml in serum) as well as patients with a disease duration longer than five years were included. The exclusion criteria were: patients with any history of chronic conditions such as liver disease, diabetes mellitus, respiratory disorders, cardiovascular diseases, alcohol abuse 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 treated as follows:

First Cycle of Ozone Treatment

MTX 12.5 mg, intramuscular (i.m.), once/week (every Monday from 9:00-10:00 in the morning) + Ibuprofen (400 mg, oral), one tablet each 8 h + folic acid (5 mg, oral), one tablet/day from Wednesday to Saturday. The ozone was generated by an OZOMED unit, Cuba. The 20 treatments by rectal insufflation (five/week from Monday to Friday) at 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.

At the end of the first cycle, ozone treatment was withdrawn from the therapeutic plan though RA patients continued with MTX, Ibuprofen and folic acid.

Second Cycle of Ozone Treatment

Three months after the preceding ozone treatment, patients treated in the first cycle received the second, applying the same therapeutic plan as described in the first cycle. Medical personnel were instructed to report all adverse reactions, whether described in the package circulars of the study medications or not.

Evaluation of Disease Activity

Changes in the development of the disease were determined via suitable indices of activity (clinical parameters) as well as anti-CCP antibodies and redox status determination before starting and after completing the first and second cycles of ozone application. Each patient served as their own control as our previous papers [4,10,11] (i.e. before medical ozone treatment). It is important to

state clearly that placebo control is not possible to use because ozone is generated through special equipment designed for it. On the other hand, there is no another gas with proven safety and without therapeutic effects.

The main variables considered were:

Clinical parameters: DAS28 [12], HAQ-DI and anti-CCP levels. Pain intensity using a Visual Analogue Scale (VAS) from '10' to '100'. This was classified as '10' (minimum pain intensity) and '100' (maximum pain intensity). No pain was considered as "0".

Secondary variables considered were: (a) Plasma 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.

Biochemical determinations

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

Anti-CCP antibodies were determined using an ELISA kit (DRG, DRG Diagnostics, GmbH, Germany) (sensitivity 90%, specificity 98.3% and diagnostic efficacy 95.3%) Erythrocyte Sedimentation Rate (ESR) was obtained using Westergren's quantitative method.

Redox parameters were determined by spectrophotometric methods using a reader plate (SUMA, Cuba) and a 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 10s intervals for 1 min [13]. After precipitation of thiol proteins using trichloroacetic acid 10%, reduced Glutathione (GSH) was measured according to the method of Sedlak and Lindsay [14] with Ellman's reagent [5′5 dithiobis (2-nitrobenzoic acid)10-2M (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) [15]. The Advanced Oxidation Protein Products (AOPP) were measured as the oxidation of iodide anion to diatomic iodine by advanced oxidation protein products [16]. Quantification of Total Hydroperoxides (TH) was measured by Bioxytech H2O2-560 kit (Oxis International Inc., Portland, OR, USA). Concentrations of Malondialdehyde (MDA) were analysed using the LPO-586 kit obtained from Calbiochem (La Jolla, CA).

In the case of biochemical variables, laboratory data for healthy individuals (n=60) were taken as normal reference values. This group of subjects corresponded in terms of age, sex and ethnicity with the patients enrolled in the study.

Statistical analysis

The OUTLIERS preliminary test for detection of error values was initially applied. In order to assess differences among the first and second cycle of ozone treatment for clinical variables, the Friedman non-parametric test (for more than two samples linked) was used. In order to contrast each redox variable at the end of the study between both cycles of ozone treatment, the Mann-Whitney U non-parametric test was applied. The risk of GSH decrease at the end of the study for each cycle of ozone treatment was calculated by Odds ratio and non-parametric Wilcoxon signed rank test for two linked samples. Results are presented as mean ± Standard Deviation (SD). The level of statistical significance used was at least $P<0.05$.

Results

General characteristics of the patients involved in the study

In relation to the baseline characteristics (Table 1), all patients were women with an average age of 53 ± 8 years receiving the same therapy while the evolution time of RA was 7 ± 2 years. No major ethnic differences were observed.

Demographic data	Patient Histories (n = 20)
Women (n/%)	20/100%
Age (years)	53 ± 8
Therapy	
Methotrexate (n/%)	20/100%
Ozone/Oxygen mixture (n/%)	20/100%
Ibuprofen (n/%)	20/100%
Folic Acid (n/%)	20/100%
Evolution time of the disease (years)	7 ± 2
Race	
Caucasian	11/55%
Non-Caucasian	9/45%

Table 1: Clinical picture of patients with rheumatoid arthritis who received two cycles of ozone treatment separated one of the other by three months.

Picture of clinical variables at the beginning and at the end of each cycle of ozone treatment

At the end of the first cycle of ozone treatment, a significant decrease in all clinical variables was observed. Three months after the first cycle, the period of time during which ozone was removed from the therapeutic plan, RA patients received another cycle of ozone treatment. The second cycle of ozone promoted a final decrease in all clinical variables as compared with the beginning of this cycle. Although there was a decreasing tendency at the beginning of the second ozone cycle, there were no statistic differences between the beginning of either two cycles. However, at the end of the second ozone exposure a stronger therapeutic response from patients was observed compared with the end of the first cycle. In some clinical variables this mainly involved anti-CCP levels (42 ± 1.5 vs 116 ± 23 U/L), DAS-28 (1.8 ± 0.96 vs 2.4 ± 0.7), and HAQ-DI (0.09 ± 0.02 vs 0.2 ± 0.05) respectively. γ-Glutamyl transferase activity showed a tendency to decrease in the context of the first cycle, but without statistical differences and remaining within the reference range. Although the pain variable showed no statistical differences, there was a decreasing tendency after the second cycle when compared with the first cycle (Figure 1).

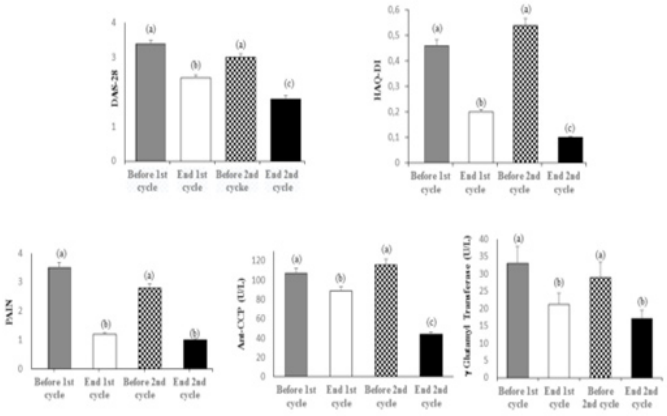

Figure 1: Medical ozone effects on clinical variables in RA patients after receiving two cycles of ozone treatment at intervals of three months between each cycle. The mean ± Standard Deviation (SD) is given. The different letters indicate statistical differences ($p<0.05$). DAS-28: Disease Activity Score 28; HAQ: DI, Health Assessment Disability Questionnaire; Anti-CCP: Anti-Cyclic Citrullinated Peptides.

Redox biomarkers at the end of both cycles of ozone treatment

The protective (antioxidants) and injury (pro-oxidants) redox markers in rheumatoid arthritis patients at the end of first and second cycles was determined in the plasma (Figure 2).

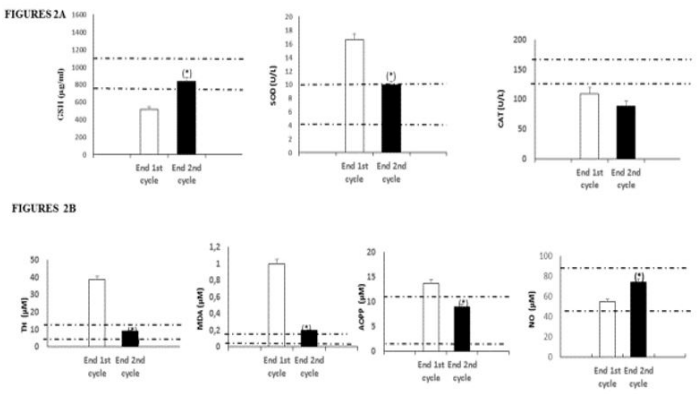

Figure 2: (A) Protective and (B) Pathogenic redox biomarkers in RA patients treated with MTX+ozone after receiving two cycles of ozone treatment at intervals of three months between each cycle. The mean ± standard deviation (SD) is given. (*) mean statistical difference $p<0.05$. Range marked with dotted lines: normal reference values (n=60 healthy subjects). GSH: Reduced Glutathion; SOD: Superoxide Dismutase; CAT: Catalase Activities; TH: Total Hydroperoxides; MDA: Malone Dialdehyde; AOPP: Advanced Oxidation Protein Products; NO: Nitric Oxide.

After the second cycle of ozone treatment, the redox response was greater than clinical responses. 85.7% of the total redox biomarkers assessed (6/7 biomarkers) returned to normal reference values. Catalase activity did not change and remained below the normal reference range.

In relation to antioxidant defense biomarkers (Figure 2A), there was a greater increase in reduced glutathione (GSH) so that it could achieve the normal reference value, whereas SOD activity showed a considerable decrease and enzymatic activity returned to normal levels.

Injury markers showed impressive changes in line with the above results. Total Hydroperoxides (TH) and lipid peroxidation (MDA) decreased their high levels and reached the normal reference values, as well as the Advanced Oxidation Protein Products (AOPP). Although Nitric Oxide (NO) remained within normal value ranges at the end of both cycles, the NO levels were better in the second cycle.

Reduced glutathione is an important participant in the endogenous antioxidant defense system, and it is depleted in RA. The results of the Wilcoxon test showed a significant GSH increase ($p<0.05$) at the end of second ozone exposure in RA patients (15/20 patients increased GSH levels at the end of second cycle of ozone treatment expressed as the difference GSH_2-GSH_1 (GSH at the end of the 2nd versus GSH at the end of the 1st treatment cycles). Only 4 patients had decreased GSH levels compared with the end of first cycle, and one patient had the same GSH concentration at the end of both cycles (Figure 3).

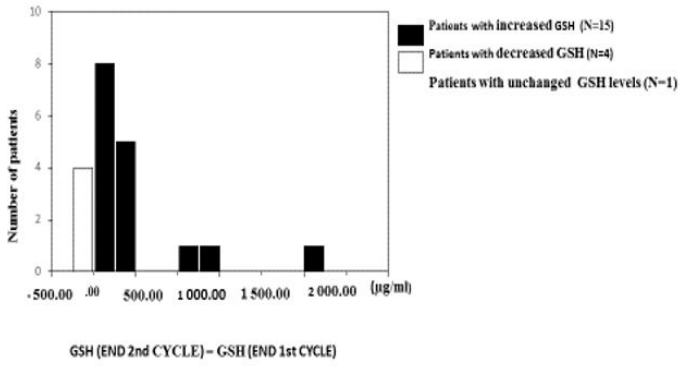

Figure 3: Increase of the GSH level (µg/ml) in 15 of 20 patients at the end of second cycle of ozone treatment, expressed as the difference GSH_2-GSH_1 (GSH at the end of the 2nd versus GSH at the end of the 1st treatment cycle). A slight decrease was found in 4 patients and no difference in 1 patient after the second treatment cycle (non-parametric Wilcoxon signed rank test for two linked samples).

A low risk of GSH concentration decrease was found at the end of the second cycle of ozone treatment (0.667), whereas the probability of GSH remaining within the normal reference interval increased by a factor of 4 (Table 2).

Risk estimation of GSH concentrations decrease at the end of the cycles	Value
Odds ratio for the treatment cycle variable (2nd cycle/1st cycle)	6.000
For cohort of GSH levels in normal reference values at the end of the cycle	4.000
For cohort of GSH levels below of normal reference value at the end of the cycle	0.667
Number of valid subjects	40

Table 2: Analysis of GSH levels at the end of second cycle of ozone compared with first cycle of treatments.

Discussion

A second ozone exposure in RA patients treated with MTX+ozone combined therapy promoted powerful clinical and redox responses. A reduction in inflammation (DAS-28) and disability (HAQ-DI) was found. These results agreed with the decrease in Anti-CCP levels at the end of the second ozone cycle. Anti-CCP antibodies are actively produced or enriched at the site of inflammation (joints and synovial tissue) and are able to participate actively in the pathogenesis of anti-CCP positive RA by enhancing oxidative stress in the rheumatoid joint [17]. Indeed, high titers (titers US, titres BRIT) of anti-CCP antibodies have been associated with an erosive disease course and outcome in RA [18]. γ-Glutamyl Transferase (GGT) concentrations were within the normal reference interval and corresponded to the decrease in anti-CCP levels combined with a clinical and redox improvement of the patients. This enzyme is close connected with the bone destruction observed in RA as mediated by excessive osteoclastogenesis. GGT acts as an endogenous activator of this type of pathological osteclastogenesis, independently of its enzymatic activity. The underlying mechanisms involve Toll-like Receptor 4 (TLR4) which recognizes GGT in order to activate inflammation-associated osteoclastogenesis [19]. The impact of the second ozone exposure on the cellular redox status was greater than the clinical response described above. All redox biomarkers returned to their normal reference interval, except the catalase activity combined with a reduction in the mediators contributing to cartilage and bone injury (TH, MDA, AOPP). These results were expected because the major ozone targets are the redox markers determining antioxidant/prooxidant cellular balance, meaning that the main effect of the second ozone exposure should be on the redox status of the patients as found in this study. It is important to emphasize the complete recovery of GSH as well as the low risk of GSH depletion found after the second ozone exposure. This antioxidant tripeptide is depleted by GGT and it has been proposed as a mediator of signal transduction events associated with regulation of cellular redox balance [20].

The clinical and redox improvement achieved in RA patients treated with combined MTX+ozone therapy suggest that a protective effect took place in the first ozone cycle involving events associated with the activity of the innate immune system such as reduction of oxidative damage, Toll-like receptor 4 activation by GGT, cytokine release, etc. After three months, a second ozone exposure is a stimulus capable of activating the innate memory that strengthens the protective responses against cartilage and bone injury associated with autoimmune disease. On the other hand, memory-induced hyper response may be involved in the pathological sequelae of innate immunity/inflammation as seen in autoimmunity [21].

Last year, important discoveries changed our angle of view in the context of the innate immune system. Features such as specificity and memory, the main traits of the adaptive immune system, are now also to a certain extent included in the context of innate immunity. The discovery of PRRs (Pattern Recognition Receptors) such as Toll-like receptors makes it possible to recognize that innate immune cells can identify different pathogenic stimuli based on their recognition of damage-associated molecular patterns. A very interesting notion is that the innate memory is, at least in part, nonspecific. This implies that an improved defensive response can be obtained by pre-challenging the host with (almost) any kind of agents [22].

The mechanisms underlying innate immune memory have not yet been fully elucidated. Nevertheless, a number of principal events were involved [22] which are closely related to the effects of medical ozone.

TLR4 represents one of the best-characterized PRRS; these are able to recognize bacterial Lipopolysaccharide (LPS) and types of molecular formation known as Damage-Associated Molecular Patterns (DAMPs) [23]. These are closely associated with the induction and development of sterile inflammation observed in diseases such those in an autoimmune context [24].

Ultralow LPS stimulation (implying a long-term slow infection with tissue debilitation) induces an innate memory that results in an enhanced reactivity to subsequent stimuli, necessary for the adequate defence of a weakened tissue [25]. The activity of GGT as regulated by medical ozone [5] may be similar to that of LPS, as LPS stimulates TLR4 and is known to exert a potent osteoclastogenic activity through this receptor [26]. It appears that extracellular GGT is able to activate other types of TLR4-expressing cells in addition, including osteoblasts and macrophages, inducing them to react with pro-inflammatory responses in a way similar to that described for LPS.

Although the underlying mechanism by which extracellular GGT can be released to serve as a TLR4-stimulating DAMP remains unclear, the production and release of GGT from the lymphocytes or inflammatory cells may be stimulated by the inflammation when accompanied by oxidative stress. Previous results have shown that the transcription of GGT is highly sensitive to oxidative stress [27], and that elevated humoral GGT levels have been observed in those diseases associated with chronic inflammation, as found for example in the synovial fluids collected from the arthritic joints [28].

Another mechanism associated with an innate immune memory is found in the type of cells involved. Among the innate immune cells, the most active innate memory cells consist of monocytes/macrophages and NK cells. In addition, innate memory can be induced at the immune stem cell level, i.e. in bone marrow niches in which non-immune cells probably contribute to the induction of stem cell priming [29]. The recent findings that epithelial stem cells retain a memory of previous inflammatory challenges by displaying an enhanced wound healing capacity after skin damage reveal that an innate memory may not be restricted to immune cells [30].

It is very interesting to find that monocytes/macrophages are able to develop different types of memory depending on the type of priming. Thus, monocytes and/or macrophages can develop a memory that makes them less reactive to certain challenges (tolerance, the avoidance of extensive tissue damage) or to an enhancement of their response (training, improved tissue surveillance e.g. against immune diseases). These different types depend on the nature of the first challenge. A low stimulation, similar to that by which medical ozone is administered (low dose), promote an enhanced reactivity to subsequent stimuli [31].

Regulation of a cytokine pattern when mononuclear cells in peripheral blood are activated by medical ozone has been demonstrated [32]. The release of Interleukin 4, 6 and 10, tumour necrosis factor alpha (TNF-α) and IFN-α and IFN-γ was dependent on ozone concentrations and incubation time of the cells. Recently, it also has been demonstrated that cytokine combinations can induce memory-like properties in human cells [22,33,34]. A parallel between innate immune memory and its relationship with medical ozone effects associated with immune system are shown in Table 3.

Innate Immune Memory	**Ozone effects and its relationship with immune response**
Memory-dependent enhancement in order to improve tissue surveillance and protection in situation of weakness [22].	Regulation of redox balance and enhancement of clinical response improve weakness and frailty in Rheumatoid Arthritis (RA) patients treated with Methotrexate+medical ozone [4].
Enhanced responses to a second challenge (priming) is synonymous of innate memory, not restricted or limited to the enhancement of innate protective responsiveness.	Second ozone exposition promoted a potentiation of clinical and redox responses stronger than the first challenge mainly on redox endogenous system which is the major targets of medical ozone.
Toll-like receptor 4 is a Pattern Recognition Receptor (PRR) which is involved in innate immune memory through its activation by damage-associated molecular pattern [23].	Ozone regulates extracellular γ-Glutamyl Transferase (GGT) in RA patients. GGT is an activator of Toll-like receptor 4 and it may be considered as Damage-Associated Molecular Patterns (DAMPs) able to develop sterile inflammation observed in the diseases such as autoimmune diseases [5,23].

Ultralow LPS stimulation (implying a long-term slow infection with tissue debilitation) induce an immune memory that results an enhanced reactivity to subsequent stimuli, necessary for adequate defense of a weakened tissue [25].	Medical ozone is administered to low dose [20] able to regulate immune system through mediators and nuclear factors mainly NF-κB [2].
Different patterns of cytokines release may be involved in the systemic establishment of a memory phenotype [22]. Cytokine combinations can induce memory-like properties in humans [33].	Regulation of cytokine combinations when peripheral blood mononuclear cells are activated by medical ozone has been shown [32]. Control of interleukin 4, 6, 10, tumour necrosis factor alpha (TNF-α), and IFN-α and γ levels was dependent of ozone concentrations and the incubation time of the cells.
Pre-conditioning is a terminology which has been used in order to identify innate immune memory.	Ozone oxidative preconditioning is the terminology in order to identify ozone capacity to regulate cellular redox balance [1].

Table 3: Parallel between innate immune memory and its relationship with medical ozone effects.

In summary, RA patients treated with combined MTX-ozone therapy increased clinical and redox response after the second cycle of ozone exposure when compared with the results from the first ozone cycle. This potentiation seems to be mediated by an innate immune memory based on the close relationship between the targets revealing an innate immune memory and the regulating effect of medical ozone.

At present, an investigation of those epigenetic markers which have been identified in association with the acquisition of an innate immune memory is in progress.

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