

Medical Ozone Reduces the Risk of γ -Glutamyl Transferase and Alkaline Phosphatase Abnormalities and Oxidative Stress in Rheumatoid Arthritis Patients Treated with Methotrexate

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Keywords Medical ozone; Methotrexate; γ -Glutamyl transferase; Oxidative stress; Rheumatoid arthritis

Abbreviations MTX: Methotrexate; RA: Rheumatoid Arthritis; DMARD: Disease-Modifying Antirheumatic Drug; PG-PS: 10S fraction of Peptidoglycan-Polysaccharide; DAS-28: Disease Activity Score 28; HAQ-DI: Health Assessment Questionnaire-Disability Index; AST: Aspartate Aminotransferase; ALT: Alanine Aminotransferase; ALP: Alkaline Phosphatase; GGT: γ -Glutamyl Transferase; AOPP: Advanced Oxidation Protein Products; NO: Nitric Oxide; TH: Total Hydroperoxides; MDA: Malondialdehyde; GSH: Reduced Glutathione; CAT: Catalase; SOD: Superoxide Dismutase; VAS: Visual Analogical Scale

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Abstract

Background: Methotrexate (MTX) + Medical Ozone increase MTX clinical efficacy in Rheumatoid Arthritis (RA) patients.

Aim: The purpose of this study was to investigate whether medical ozone could decrease the risk of γ -Glutamyl Transferase (GGT), Alkaline Phosphatase (ALP) abnormalities and oxidative stress in RA patients.

Methods: A prospective study with 100 patients was performed, who were divided into two groups: one (n = 50) treated with MTX, Folic acid and Ibuprophen (MTX Group) and the second group (n = 50) receiving the same as the MTX Group + medical ozone by rectal insufflation. The diagnosis of RA patients was performed using Anti-Cyclic Citrullinated Peptides levels, DAS-28 and HAQ-DI. The risk of liver marker abnormalities and the oxidative stress were evaluated by means of biochemical methods and statistical tests.

Results: MTX + Ozone reestablished γ -Glutamyl Transferase (GGT), reduced Alkaline Phosphatase (ALP), enhanced the antioxidants endogenous and decreased oxidative damage to biomolecules with regard to MTX monotherapy. Patients treated with MTX + medical ozone decreased the risk of GGT and ALP abnormalities by a factor of 4. An inverse correlation between GGT and reduced glutathione was found.

Conclusions: MTX + Ozone regulated and decreased the risk of GGT and ALP abnormalities. The modulation of GGT by ozone and the reduction of oxidative stress may play an important role against liver damage induced by MTX.

Introduction

Methotrexate (MTX) is the anchor Disease-Modifying Antirheumatic Drug (DMARD) in Rheumatoid Arthritis (RA) treatment. It is used in monotherapy and/or in combination with other synthetic or biological DMARDs. However, in spite of its beneficial effects, its toxicity is responsible for interrupting long-term treatment due to the occurrence of MTX-related adverse drug reactions, which are the main cause of drug withdrawal [1].

The mechanisms underlying methotrexate hepatotoxicity are unclear. A large variety of histological liver lesions including dystrophic nuclei, macrovesicular steatosis, cell necrosis, cholestasis, Ito cell hyperplasia, portal inflammation, liver fibrosis and even cirrhosis have been reported. Another theory is that the prolonged intracellular accumulation of MTX, especially MTX polyglutamates, causes a prolonged depletion of folate, which is required for DNA synthesis. Although these events are considered the most MTX-specific lesions, their real incidence is not really known [2,3].

Medical ozone is a regulator of cellular redox balance as well as other molecules. On account of its oxidative pre/post conditioning mechanism [4] it has achieved clinical efficacy in oxidative etiology diseases such as diabetic foot [5], disc hernia [6], and different experimental models. Moreover, medical ozone has been able to reduce inflammation, IL-1 β , TNF- α mRNA levels and oxidative stress in PG/PS-induced arthritis in rats [7] while MTX + medical ozone combined therapy increased clinical efficacy as compared with MTX monotherapy in RA patients. The reduction of inflammation, pain, disability and anti-CCP (anti cyclic citrullinate peptides) levels was achieved [8].

Previously, ozone's protective effects against liver damage such as MTX-induced hepatotoxicity in rats [9], CCl_4 -induced liver damage and hepatic ischemic reperfusion injury [10,11] have been demonstrated. These results were the basis for this work for which reason the aim of this study was to investigate whether MTX + medical ozone combined therapy was able to decrease the risk of GGT, ALP abnormalities and oxidative stress in RA patients treated with MTX. The role of MTX + medical ozone on GGT activity, oxidative stress and the relationship of these events with the protection against hepatic injury were subjects of the study.

Materials and Methods

Study design

A prospective study in RA patients was here carried out. It 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 principle of Helsinki's Declaration 2008 [12]. All patients gave their informed consent to being enrolled after receiving adequate information about the study (characteristics of the study, benefits and possible side effects). Before enrolling, 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 [13].

For the calculation of the size of the sample, the Medstat Systems, Inc. (version 2.1, 1989; Fridley, MN, USA) was used. The statistical difference at the beginning and at the end of ozone therapy was 0.2 with type 1 error of 0.05 [14]. The target level of enrollment was determined at 45 patients. Assuming that 10% of studied patients would be lost to follow-up, 50 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 [15] criteria for RA (morning stiffness, swelling of hand joints, swelling of three or more joints, and symmetric swelling of joints) were eligible to participate in the study. Patients of the National Institute of Rheumatology, Ministry of Public Health, Cuba with Disease Activity Score 28 ($\text{DAS}_{28} > 3.2$), Anti-Cyclic Citrullinate Peptides (anti-CCP > 10 U/ml in serum), Health Assessment Questionnaire-Disability Index: (HAQ-DI, according to the Spanish validated version) [16], disease duration and time of treatment with MTX at least more than two years given that MTX-induced hepatotoxicity is considered an accumulative process significantly related to the occurrence of liver injury associated with the exposure time to MTX [17]. Examination of the patients was achieved by blinded physician different to the one selecting the patients according to a randomized scheme of treatment and a preliminary brief medical history. Exclusion criteria were: patients with any previous history of chronic disease such as liver diseases, diabetes mellitus, respiratory disorders, cardiovascular and renal diseases. Alcohol consumption and smoking were not included in the study as they are risk factors capable of increasing the hepatotoxic effects of MTX. 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, 12.5 mg, intramuscular (im), one time/week (each 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 equipment, Cuba. Ozone was obtained from medical grade oxygen, was used immediately as generated and it represented only about 3% of the gas (O_3/O_2) mixture. The ozone concentration is measured by using a built-in UV spectrophotometer at 254 nm (accuracy, 0.002A at 1 A, repeatability 0.001A and calibrated with internal standard). 20 treatments by rectal insufflations (five/week from Monday to Friday). 25 mg/l to 40 mg/l of ozone in graded fashion and in increasing order was 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.

Evaluation of liver markers

Changes in the evolution of liver tests as well as redox status determinations at the end of clinical study (21 days) were assessed. Each patient was its own control (i.e. prior to Medical Ozone treatment).

The main variables considered were:

Liver markers: Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Alkaline Phosphatase (ALP), γ -Glutamyl Transferase (GGT) activities and serum albumin were determined using HITACHI equipment (Japan). Abnormalities in liver markers were defined as above normal laboratory range (AST < 40 IU/l; ALT < 40 IU/l; ALP 40-190 IU/l; GGT 8-36 IU/l; albumin 35-50 g/l).

Secondary variables considered were: (a) Serum levels of injury markers as Advanced Oxidation Protein Products (AOPP), fructolysine, Nitric Oxide (NO), Total Hydroperoxides (TH) and Malondialdehyde (MDA). (b) Serum levels of protective redox markers as reduced Glutathione (GSH), Catalase (CAT) and Superoxide Dismutase (SOD) activities. (c) Side effects.

A result was considered to be good when GGT, ALP and transaminases activities decreased or had not increased by the end of the study as compared with the beginning. An increase in endogenous antioxidants (GSH, SOD and CAT) and a decrease in injury redox markers (NO, AOPP, fructolysine, TH and MDA) were also considered as good results.

The protective effects against MTX-induced hepatotoxicity was considered to be successful if $\geq 70\%$ of the patients treated with MTX + medical ozone had better outcome when compared with MTX, taking the above variables into account.

Biochemical determinations

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

Anti-CCP antibodies were determined by ELISA (DRG, DRG Diagnostics GmbH, Germany) (sensitivity 90%, specificity 98.3% and diagnostic efficacy 95.3%).

Redox parameters were determined by spectrophotometric methods using a plate reader (SUMA, Cuba) and 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 [18]. After precipitation of thiol proteins using trichloroacetic acid 10%, reduced glutathione (GSH) was measured according to the method of Sedlak and Lindsay [19] 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) [20]. The Advanced Oxidation Protein Products (AOPP) was measured as the oxidation of iodide anion to diatomic iodine by advanced oxidation protein products [21]. Relative fructosylsine content (Amadori's product of glycated serum protein) was measured by using the redox indicator NBT at 530 nm [22]. Quantification of Total Hydroperoxides (TH) was measured by Bioxytech H₂O₂-560 kit (Oxis International Inc., Portland, OR, USA) Concentrations of Malondialdehyde (MDA) was analyzed using the LPO-586 kit obtained from Calbiochem (La Jolla, CA).

Statistical analysis

In order to identify the variables capable of distinguishing the RA patients' changes in accordance with the treatments (MTX or MTX + medical ozone) canonical discriminant analysis was used. Comparisons of each variable (before the beginning and at the end of the study) for each treatment were assessed using the Wilcoxon signed rank test and Student t-test for correlated samples whereas, in order to contrast each variable at the end of the prospective study with regard to the treatment, the Mann-Whitney U and Student t-tests for independent samples were used. The risk of abnormalities in GGT and ALP activities at the end of the study for each treatment were estimated in the case of GGT using the Cochran and Mantel-Haenszel tests. As regards ALP, the Relative Risk (RR) or Odds ratio was calculated. Correlation between GSH and GGT was evaluated by means of Pearson's coefficient.

Results

General characteristics of the patients involved in the study

In relation to the baseline characteristics (Table 1), both groups were similar at randomization ($P > 0.05$). In the sex context, women were predominant. No differences between groups (MTX and MTX + Ozone) with regard to previous therapy, evolution time of the disease and time of treatment with MTX were found, whereas patients in

the MTX + medical ozone group displayed differences in age when compared with MTX group. In the MTX group, Caucasians were predominant. The opposite was the case with the MTX + medical ozone group.

MTX Group: Methotrexate + Ibuprophen + Folic Acid. MTX + Ozone Group: Same Group MTX + Medical Ozone.

Age and Evolution time of disease and time of treatment with MTX data represent the mean \pm SD of each group. Means with different letters indicate significant differences ($P < 0.05$) between both groups

Liver tests in MTX and MTX + medical ozone groups

Compared with the beginning of the study, GGT activity had increased by its (40.39 ± 7.33 vs. 36.55 ± 8.7 IU/l, respectively) in RA patients treated with MTX monotherapy. In the case of MTX + medical ozone the GGT had decreased by the end compared with the beginning of the prospective study (26.49 ± 7.62 vs. 38.97 ± 13.34). In addition, this treatment brought GGT back to its reference range (26.49 ± 7.62 , reference range: 8 - 36 IU/l). Differences between both treatments could be found at the end of the study (40.39 ± 7.3 vs. 26.49 ± 7.62 IU/l). ALP decreased its activity in the MTX + ozone group compared with MTX at the end of the study (208.25 ± 50.35 vs. 230.68 ± 57.72). Although transaminases changed between the treatments they stayed in reference range whereas albumin didn't change.

Given that GGT and ALP activities were the markers for liver damage showing differences with regard to the type of treatment (MTX or MTX + medical ozone), the risk of liver injury from GGT was estimated using the Cochran and Mantel-Haenszel tests, while the Relative Risk (RR) or Odds ratio was calculated for ALP. These estimations showed that RA patients treated with MTX alone had a relative risk of liver disorders approximately 4 times higher than in those patients treated with MTX + medical ozone. MTX/MTX + medical ozone: γ glutamyl transferase 3.8 (95% CI 1.37-10.58 and ALP 4.0 (95% CI 1.2 - 17.44) (Figure 1).

Table 1: Clinical picture of patients with Rheumatoid Arthritis.

Demographic data/patients histories	MTX Group (n=50)	MTX+ Ozone Group (n=50)
Women (n/%)	45/90	5/90
Men (n/%)	56 \pm 7 ^(a)	52 \pm 8 ^(b)
Age (years)	56 \pm 7 ^(a)	52 \pm 8 ^(b)
Previous therapy		
(Methotrexate) (n/%)	50/100	50/100
Corticosteroids	0	0
Evolution time of disease (years)	9 \pm 9 ^(a)	11 \pm 10 ^(a)
Time of treatment with MTX (years)	4.56 \pm 4.8 ^(a)	0.57 \pm 4.7 ^(a)
Race		
Caucasian (n/%)	26/52	22/44
Non-Caucasian (n/%)	24/48	228/56

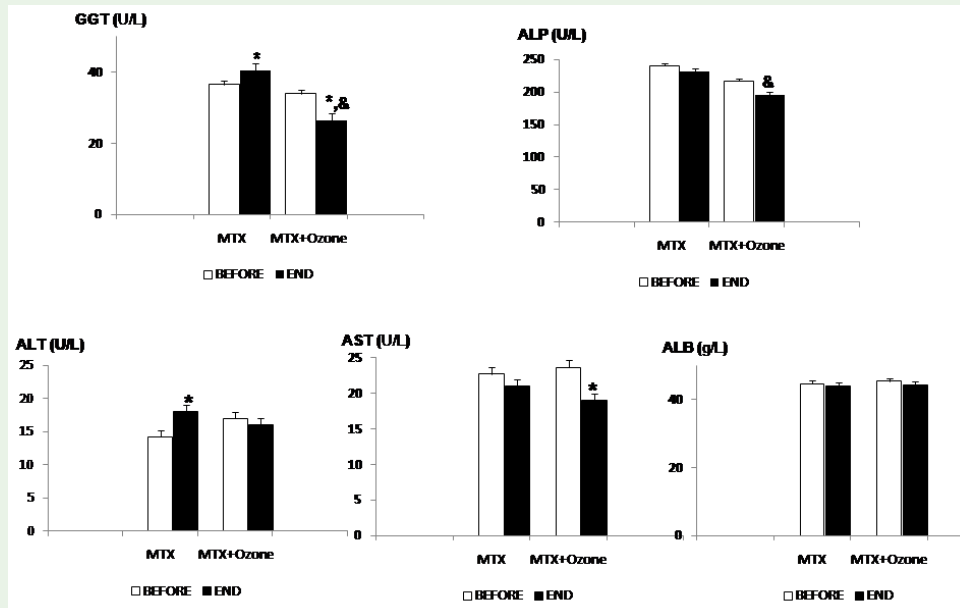


Figure 1: Liver tests of rheumatoid arthritis patients in MTX and MTX + Ozone groups. γ -Glutamyl Transferase (GGT), Alkaline Phosphatase (ALP), Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST) Activities and Albumin (ALB). "Before" means before the beginning and "End" 24 hours after the last ozone treatment (conclusion of the prospective study). Normal Laboratory Range (AST < 40 IU/L; ALT < 40 IU/L; ALP 40-190 IU/L; GGT 8-36 IU/L; Albumina 35-50 g/L). Data represent the mean \pm SD of each group. *P < 0.05 "End" vs. "Before" (&) P < 0.05 "End" MTX+Ozone vs. "End" MTX. Wilcoxon signed rank test and Student t-test for correlated samples were used.

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 investigated.

MTX + medical ozone improved the efficacy of the antioxidant endogenous system against oxidative injury, resulting in a decrease in damage to biomolecules (lipids and proteins) as well as in TH levels and nitric oxide concentrations. By contrast, patients who received no ozone were found to have a reduced antioxidant defense capability, simultaneously showing a higher level of damage (Figure 2A,B).

Assessment of the influence of MTX + medical ozone treatment on GGT, ALP and redox markers associated with liver damage in RA patients

Table 2 showed that RA patients treated with MTX + medical ozone displayed a larger number of patients in percent with positive changes in the markers for liver injury and redox status with regard to MTX monotherapy. In addition, MTX + medical ozone increased the number of RA patients who improved in all markers, whereas the opposite was observed in MTX monotherapy. In the MTX group, there was an increase in the number of patients above the reference ranges for GGT, CAT, TH, Fructosylamine and NO at the end of the prospective study.

In order to clarify whether there was any relationship between

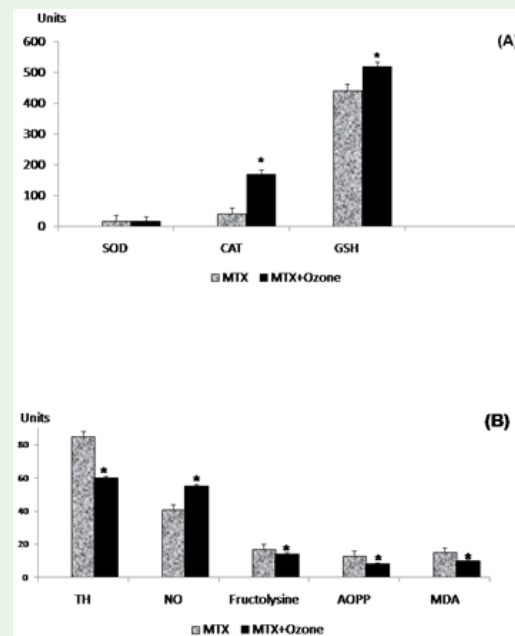


Figure 2: Redox status of RA patients in MTX and MTX + Ozone groups at the "End" of the study. "End" means 24 hours after the last ozone treatment (finish of the prospective study). (A) Protective, (B) Injury redox markers. Units of each marker are: SOD (U/ml/min) and CAT (U//min) activities, GSH (μ M), TH (μ M), NO (μ M), Fructosylamine (relative fructosylamine content), AOPP (μ M), MDA (μ M). Data represent the mean \pm SD of each group. *P < 0.05 MTX + Medical ozone vs. MTX. Mann-Whitney U and Student t-test for independent samples were used.

Table 2: Changes in liver and redox markers associated with hepatic injury in RA patients treated with MTX and MTX + ozone at the beginning and at the end of the prospective study (n/%).

Markers	MTX		MTX+Ozone		Changes in GGT, ALP and redox status of RA patients at the end of study ^(b)	
	Before the Beginning ^(a)	At the end ^(a)	Before the Beginning ^(a)	At the end ^(a)	MTX	MTX + Ozone
Liver Tests						
GGT	13/26%	17/34%	10/20%	2/4%	66%	96%
ALP	24/48%	23/46%	20/40%	16/32%	54%	68%
Redox Markers						
GSH	18/36%	18/36%	18/36%	15/30%	64%	70%
CAT	9/18%	12/24%	8/16%	1/2%	76%	98%
TH	16/32%	17/34%	17/34%	11/22%	66%	78%
MDA	18/36%	15/30%	15/30%	12/24%	70%	76%
AOPP	10/20%	9/18%	10/20%	5/10%	82%	90%
Fructosylamine	12/24%	14/28%	10/20%	8/16%	72%	84%
ON	12/24%	13/26%	9/18%	9/18%	74%	82%

- a. Mean quantity and percent of patients with marker levels over reference range "before the beginning" and "at the end" of the prospective study.
- b. Mean percent of patients who achieved the reference range or the marker moved toward normal interval -at the end of the prospective study.

MTX (Methotrexate), GGT (γ -Glutamyl Transferase), ALP (Alkaline Phosphatase), reduced glutathione (GSH), CAT (Catalase), TH (Total Hydroperoxides), MDA (Malondialdehyde), AOPP (Advanced Oxidation Protein Products), ON (Nitric Oxide).

reduced glutathione and GGT activity in the patients, correlations between both variables were evaluated. 70% of RA patients displayed an inverse lineal correlation ($r = -0.61$, $P = 0.013$).

Side effects were not observed, neither was MTX withdrawal necessary during the prospective study.

Discussion

Canonical discriminate statistical analysis ($P < 0.05$) demonstrated that clinical and redox variables selected in order to characterize the response of the patients made it possible to distinguish the outcome according to the treatment they were receiving (MTX or MTX + medical ozone). MTX + medical ozone reduced the risk of MTX-induced GGT and ALP abnormalities. RA patients treated with combined therapy improved the outcome in $\geq 70\%$ with regard to GGT, ALP and redox markers compared with patients treated with MTX alone (Table 2). Inverse correlation between GGT and GSH underlines the role of γ -glutamyl cycle and GSH homeostasis in RA patients.

Though the MTX-induced hepatotoxicity mechanism is unclear, the increase in GGT and ALP activities (Figure 1) together with the depletion of the antioxidant endogenous system (GSH and CAT) and the increase in lipid peroxidation (MDA) (Figure 2) suggest that the patients treated with MTX alone may be in risk of liver injury (cholestasis and fibrosis). Clinically, the cholestatic patients present pruritus (itching), fatigue and, in severe forms, jaundice, which is reflected by elevated serum bilirubin levels. In the early stages of the condition, symptoms might be absent and only increased serum activities of Alkaline Phosphatase (ALP) and/or γ -glutamyl-transferase (γ -GT) indicate a cholestatic condition [23]. On the other hand, methotrexate therapy in patients with RA has been shown to rise plasma homocysteine levels. Excess homocysteine can generate oxidative stress (depletion of antioxidants and increase of

malondialdehyde) [24] or sensitize the cell to its cytotoxic effects. Homocysteine has been shown to induce Endoplasmic Reticulum (ER) stress which, when unresolved, leads to fatty infiltration of the liver. In addition, it can also activate proinflammatory cytokines. The combination of these insults could contribute to the activation of hepatic stellate cells, which leads to liver fibrosis [25]. Moreover, cholestasis and fibrosis, as liver injury induced by drugs, share common cellular effects such as inflammation and oxidative stress [26]. These pictures are similar to those displayed by RA patients treated only with MTX monotherapy in this prospective study.

The inclusion of medical ozone in the combined therapy (MTX + medical ozone) reestablished GGT activity to its reference interval. Increase of GGT plays an important role in GSH exhaustion. GSH depletion may be an underlying factor for liver toxicity and this cytotoxicity may be followed by cell death, by either apoptosis or necrosis [27]. Moreover, high intracellular ROS levels accompanied by GSH depletion, lipid peroxidation, protein oxidation/alkylation and respiratory complex changes are associated with liver mitochondrial dysfunction which is a critical factor in many types of chronic liver diseases [28].

Dysregulation of GGT and GSH contributes to the pathogenesis of many chronic pathological conditions, and many of them are considered as RA comorbidities. These include diabetes mellitus, pulmonary and liver fibrosis, cholestatic liver injury, endotoxemia and drug-resistant tumor cells [29]. Patients treated with MTX monotherapy showed an increased oxidative stress. This redox status was associated with an increment of GGT, decrease of GSH and increase of lipid peroxidation and protein damage. These results were in line with other studies in which cartilage and bone destruction, damage to hyaluronic acid, lipid peroxidation products and oxidative damage to proteins have been demonstrated [30]. By contrast, MTX + medical ozone improved the cellular redox balance with emphasis on the reestablishment of GGT and the increase of GSH levels.

On the other hand, epidemiologic studies have shown inverse associations between antioxidant levels and RA [31]. In this prospective study, an inverse linear correlation between GSH and GGT has been found. These results are in line with the improvement of cellular redox balance in RA patients treated with MTX + medical ozone.

GGT regulation by ozone was an interesting finding. To our knowledge, it is the first study in which medical ozone regulation of GGT activity is shown. At present, GGT is considered to be a risk marker in chronic diseases with a high morbi-mortality [32]. The close inverse relationship between GGT and GSH has led to consider GGT as a marker of oxidative stress [33,34].

Finally, after four weeks of treatment with medical ozone, MTX clinical efficacy was not modified and no adverse reactions were detected. Although MTX + medical ozone improved RA patients outcome disease remission still wasn't completely achieved (DAS_{28} , 3 ± 0.2 and HAQ-DI, 0.3 ± 0.1 , low activity) therefore it was necessary a second cycle of treatment which is in progress.

In summary, MTX + medical ozone reduced the risk of MTX-induced GGT and ALP abnormalities in RA patients under our experimental conditions. Medical ozone regulation at GGT levels demonstrates the relationship between an increase in GSH, improvement of cellular redox balance and the reduction of liver injury in RA patients

Studies involving MTX + medical ozone effects are in progress. These will consist of a second cycle of ozone treatment in RA patients as well as investigations of the mechanism involved in GSH, and GGT beneficial effects.

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