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Ozone Therapy Effects on Biomarkers and Lung Function in Asthma

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Background. The relationship and behavior of serum immunoglobulin E (IgE) level, peripheral blood mononuclear cell (PBMC) human leukocyte antigen DR (HLA-DR) expression and erythrocyte glutathione antioxidant pathway in asthma patients treated with systemic ozone therapy have not been studied before.

Methods. Asthma patients were treated about 1 year with three cycles (5 or 6 months each) with three different ozone therapy protocols. Ozone major autohemotherapy (MAHT) was applied at doses of 4 and 8 mg, 15 sessions each cycle; and ozone rectal insufflations (RI) at a dose of 10 mg, 20 sessions each cycle. Serum IgE, HLA-DR expression in PBMC and biomarkers for antioxidant pathway were measured before and at the end of each cycle. Lung function and symptoms test were recorded at the beginning and after the third cycle.

Results. IgE and HLA-DR decreased with the three types of treatments, while increments in reduced glutathione, glutathione peroxidase, glutation reductase and glutathione S-transferase were achieved with all treatments. Lung function and symptoms test were markedly improved. However, in all parameters the best response was obtained in the order: MAHT at 8 mg better than MAHT at 4 mg better than RI at 10 mg. Before ozone treatment, glutathione antioxidant parameters were under the normal reference values, suggesting the occurrence of oxidative stress associated with atopic asthma.

Conclusions. This study demonstrates the effectiveness of ozone therapy in reducing IgE and inflammatory mediators along with the induction of antioxidant elements. The study raises the role of systemic ozone therapy in atopic asthma by means of its immunomodulatory and oxidative stress regulation properties.

Key Words: Oxidative stress, HLA-DR, IgE, Antioxidant pathway, FEV1, FVC.

Introduction

Allergic asthma is characterized by airway hyperresponsiveness and airway inflammation. Population studies have shown that the majority of adults and children with well-documented asthma are atopic, and the prevalence of asthma is greater in subjects with high serum immunoglobulin E (IgE) levels in a close relation with inflammatory cells (1–3). Examination of bronchoalveolar lavage (BAL) fluid cells and supernatants from allergic asthmatics has revealed the existence of Th2-like cytokine patterns (4,5) together with the IgE elevation (6). Increased IgE level in peripheral blood and bronchoalveolar tissue has been directly associated with atopic asthma pathology (7,8). Therefore, some therapeutic strategies today in atopic asthma are addressed to reduce IgE (9–11).

On the other hand, bronchial epithelial cells, lymphocytes and dendritic cells also play an active role in asthma.

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After allergen activation they release increased amounts of inflammatory mediators (12–14) and express higher levels of human leukocyte antigen DR (HLA-DR) molecule (15–17), which have been identified as a marker for eosinophil activation and therefore implicated in the cellular network underlying inflammation in asthma.

Systemic ozone therapy has been proven to be effective to modulate immune system by inducing the production of cytokines from peripheral blood mononuclear cells (PBMC) (18,19) and to regulate the oxidative stress by inducing an increase of cellular antioxidant system (20–22).

Taking into account that Th2 cytokines are pivotal in regulating the allergic phenotype, the IgE response or the inflammatory cell-mediated function (23), we explore the hypothesis that systemic ozone therapy has a beneficial role in asthma by reducing IgE level and inflammatory mediators along with a positive influence on the antioxidant defense system.

Materials and Methods

Patients and Treatments

One hundred thirteen asthmatic patients (ages: 15–50 years) including both sexes were treated by ozone therapy. They were divided into three groups. Two groups were treated by ozone major autohemotherapy (MAHT) and the other one by ozone rectal insufflations (RI). All received three cycles of treatment. Each cycle comprised 15 sessions (five a week) for MAHT using an ozone dose of 4 mg (20 μg/mL per mL of blood and 200 mL of volume) in the first group, or 8 mg (40 μg/mL per mL of blood and 200 mL of volume) in the second one. MAHT was done by using sodium citrate 3.8% in blood transfusion glass flasks. Each cycle of the third group comprised 20 sessions using an ozone dose of 10 mg (50 μg/mL and 200 mL of gas volume). Time between cycles was 5 or 6 months in all groups. No other medication was given during the study.

All patients signed a specific consent to participate in the study and the Scientific and Ethical Committee from Ozone Research Center of Cuba approved the entire project.

Determinations

Blood samples were drawn from patients before and at the end of each cycle. Red blood cells, serum and PBMC were obtained by gentle centrifugation and Ficoll-Hypaque-dextran gradient. Total serum IgE quantification was done using a commercially available immunoenzymatic ELISA kit. For HLA-DR measurement, a flow cytometry study on lymphocyte subpopulation was done using the ior dr1 (IgG2a) anti HLA class II fluorescent (FITC) monoclonal antibody. Reduced glutathione (GSH) and glutathione reductase (GR) were measured in erythrocytes using the methods of Beutler (24). Erythrocyte glutathione peroxidase (GPx) and glutathione-S-transferase (GST) activities were measured by the modification of Faraj et al. (25) to the method of Thomson (26) and by the method proposed by Habig and Jakoby (27), respectively.

Symptoms scores were recorded and respiratory function tests (forced expiratory volume at 1 sec [FEV1] and forced vital capacity [FVC]) were done using baseline spirometry tests before and at the end of each cycle.

Statistical Analysis

Analysis of the results was done using the values before starting the first cycle and those at the end of the third cycle of treatment. Results are expressed as mean ± SD. Comparison of matched samples was done by Wilcoxon test. A p value ≤ 0.05 was considered to be statistically significant.

Results

Patient Classification

Serum IgE and HLA-DR expression values before and after ozone therapy are reported in Table 1. A trend to diminish the values of both parameters was observed after ozone therapy because only HLA-DR with MAHT (8 mg) decreased statistically significantly. Before treatment, average IgE levels were 193 ± 73 IU/mL for MAHT (4 mg), 225 ± 87 IU/mL for MAHT (8 mg), and 196 ± 52 IU/mL for RI (10 mg), whereas HLA-DR averages were 32 ± 12% for MAHT (4 mg), 27 ± 12% for MAHT (8 mg), and 29 ± 12% for RI (10 mg). Very high abnormal values were not present in any of the three groups, indicating that all patients did not have the same asthma stage; therefore, they would not respond equally to the treatment.

Consequently, a distribution according to serum IgE level and PBMC HLA-DR expression was done (Table 2). Patients with very high figures in both parameters comprised only 43%. The rest of the patients had at least one of these parameters below the cut-off levels. We have established high cut-off levels of 250 IU/mL for IgE and 35% for HLA-DR; however, other authors assume cut-off

Table 1. Values of serum IgE and HLA-DR expression in PBMC before and after ozone therapy for the entire number of patients

	IgE (IU/mL)		HLA-DR (%)	
Treatment	Before	After	Before	After
MAHT (4 mg) (n = 35)	193 ± 73	194 ± 81	32 ± 12	29 ± 8
MAHT (8 mg) (n = 41)	225 ± 87	188 ± 78	27 ± 10	21 ± 2*
RI (10 mg) (n = 37)	196 ± 52	194 ± 74	29 ± 12	27 ± 9

PBMC, peripheral blood mononuclear cells; MAHT, major autohemotherapy; RI, rectal insufflations; n, number of patients.
*$p \leq 0.05$.

Table 2. Subgroup classification according to high serum IgE (>250 IU/mL) and high HLA-DR (>35%)

Subgroup	MAHT (4 mg)	MAHT (8 mg)	RI (10 mg)	Total (%)
High IgE + high HLA-DR	18	15	16	49 (43)
Normal IgE or HLA-DR	17	26	21	64 (57)
Total	35	41	37	113 (100)

HLA-DR, human leukocyte antigen DR; MAHT, major autohemotherapy; RI, rectal insufflation.

levels between 121 and 150 for IgE (28–30), and <25% for HLA-DR (16,31). This reason is because many patients are in the normal subgroup in our study. We have established high cut-off levels in order to be sure we are evaluating a real atopic subgroup of asthmatic patients.

Ozone Therapy Effects on IgE, HLA-DR and Glutathione Antioxidant Pathway

Figures 1 and 2 show MAHT at a dose of 4 mg produced a slight, non-statistically significant decrease in IgE level (15%) and HLA-DR expression (10%) at the end of the third cycle compared with the starting values. However, MAHT at a dosage of 8 mg produced in IgE level (61%) and HLA-DR expression (57%) statistically significant decreases. Rectal insufflations also induced a significant decrease in both parameters (30% for IgE and 40% for HLA-DR).

Results for glutathione antioxidant pathway are shown in Table 3. GPx raised in a statistically significant manner with MAHT (4 mg) at the end of the third cycle of ozone treatment (8.90 vs. 11.26 IU/gHb). However, ozone therapy with MAHT (8 mg) produced significantly higher values in all of the antioxidant biomarkers (GSH = 1.78 vs. 2.86

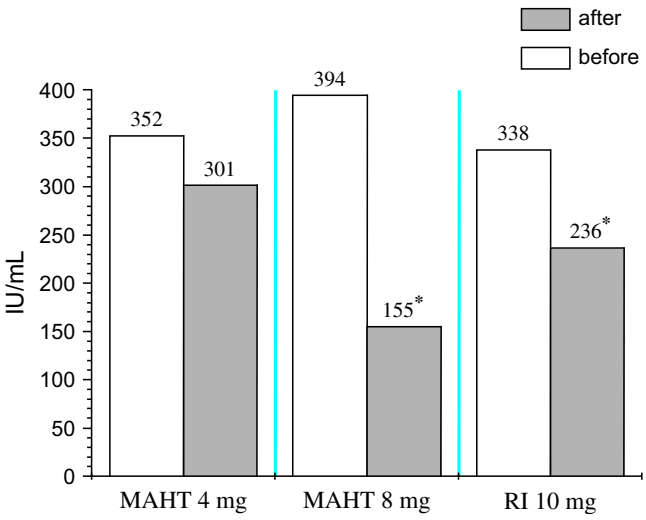

Figure 1. IgE values before first and after third cycle of ozone therapy in patients with real atopic asthma. *p <0.05.

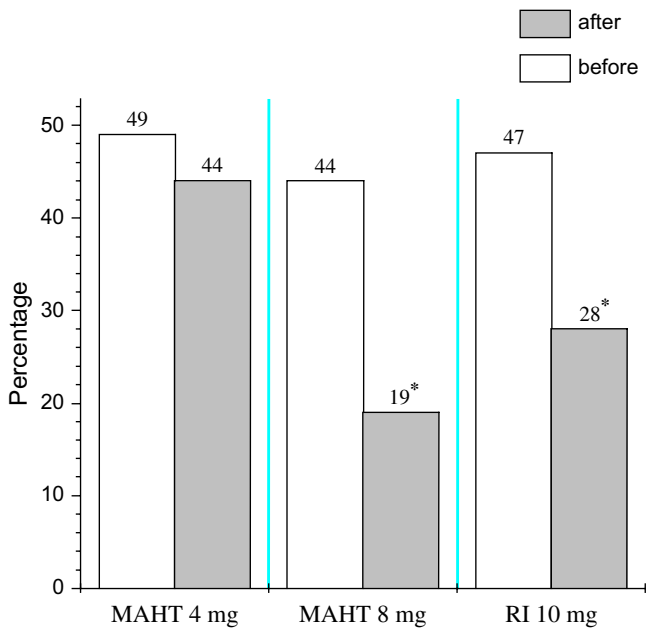

Figure 2. HLA-DR values before first and after third cycle of ozone therapy in patients with real atopic asthma. *p <0.05.

μmol/gHb; GPx = 7.56 vs. 14.21 IU/gHb; GR = 4.50 vs. 6.02 IU/gHb; GST = 5.84 vs. 9.99 IU/gHb). Treatment with RI (10 mg) stimulated remarkable increase in GPx (8.35 vs. 11.50 IU/gHb) and GST (7.01 vs. 10.08 IU/gHb) enzymes.

The changes that occurred in lung function are displayed in Table 4. No statistical changes were observed in any of the pulmonary function parameters with MAHT (4 mg). Nevertheless, a positive tendency was observed in the three parameters. MAHT-8 mg showed a significant recovery of FVC (2.34 vs. 2.99 L) and both forms of FEV_1 (1.46 vs. 2.20 L and 46.00 vs. 73.25%). A statistically significant increase was also observed for both forms of FEV_1 (1.59 vs. 1.81 L and 50.53 vs. 59.63%) when RI (10 mg) was applied. FVC was not statistically significantly increased.

Clinical improvement from episodic dyspnea, wheezing and medication was better with MAHT (8 mg) than with RI (10 mg), and better than MAHT (4 mg) (data not shown).

Discussion

In our opinion, airway hyperresponsiveness is closely related to the redox and immunological status of asthma patients; therefore, systemic ozone therapy with its properties for oxidative stress regulation and modulation of the immune system can be viewed as an efficient therapy for atopic asthma.

In the present study we observed that the three groups of ozone therapy treatments produced remarkable decreases in IgE level and HLA-DR expression. Considering the extension effect on each group, the results suggest that

Table 3. Glutathione antioxidant pathway values before and after three cycles of ozone therapy in real atopic asthma patients

	MAHT (4 mg)		MAHT (8 mg)		RI (10 mg)	
	Before	After	Before	After	Before	After
GSH	1.65 ± 0.14	1.75 ± 0.39	1.78 ± 0.22	2.86 ± 0.34*	1.80 ± 0.10	1.93 ± 0.11
GPx	8.90 ± 1.11	11.26 ± 0.8*	7.56 ± 1.40	14.21 ± 1.98*	8.35 ± 0.98	11.50 ± 1.2*
GR	3.25 ± 0.80	3.46 ± 0.24	4.50 ± 0.71	6.02 ± 0.20*	3.89 ± 0.23	3.91 ± 0.4
GST	6.55 ± 0.35	6.22 ± 0.95	5.84 ± 1.21	9.99 ± 0.80*	7.01 ± 1.01	10.08 ± 1.1*

Values for GSH are expressed in μmol/g Hb, and for GPx, GR, and GST are expressed in IU/g.Hb.
*p <0.05.

both parameters are closely related because their responses were apparently similar. Another conclusion drawn from these results is that ozone therapy effect is dependent on the applied route and ozone concentration.

It has been suggested that reactive oxygen species (ROS) play an important role in the pathogenesis of airway inflammatory diseases (32,33). In asthma patients, a correlation has been found between the degree of bronchial responsiveness to methacholine and the production of superoxide anion by peripheral polymorphonuclear leukocytes (34,35). Katsumata et al. reported that ROS could induce bronchoconstriction and airway hyperresponsiveness in anesthetized cats (36). On the other hand, glutathione and antioxidant enzymes have been found to protect the lung against ROS toxicity (37,38). These findings demonstrate that ROS exert a direct effect in mediating bronchial responsiveness and asthma. Because ozone therapy has the property to regulate the oxidative stress caused by ROS (20–22), we tried to determine if there is some relationship between asthma immunological mediators and antioxidant defense systems. One of these antioxidant defense systems is the glutathione antioxidant pathway, which includes the enzymes GPx, GST and GR, together with GSH metabolite.

In our study we found the three groups of patients had values under normal range for GSH (<2.00 μmol/g Hb) and GPx (<11.62 IU/g Hb) before ozone treatment (Table 3), suggesting the existence of oxidative stress in these patients. This is evidence for the relationship between high IgE level and the oxidative stress occurrence. After ozone treatment MAHT at a dose of 4 mg induced a significant increase in GPx enzyme activity (approximately 130%), with non-statistically significant changes in the rest of the parameters. MAHT at a dose of 8 mg influenced positively all the components of the glutathione antioxidant pathway; the major increase being in the GPx (approximately 190%), followed by GST (approximately 170%), GSH (approximately 160%), and finally GR (approximately 140%). RI at a dose of 10 mg produced significant enhancement in GPx (138%) and GST (144%) enzymes and no significant changes in GSH and GR parameters. It is evident that MAHT at ozone dose of 8 mg induced the most powerful response in stimulating the glutathione antioxidant pathway, followed by the response induced by RI at ozone dose of 10 mg. MAHT at 4 mg produced a mild response. Thus, the effect on glutathione antioxidant pathway was also dose and route dependent. This behavior was inversely similar to that seen for IgE and HLA-DR. For that reason, we assumed in these patients there was a close relationship between the asthma immunological mediators and the antioxidant system. It is important to point out that all groups of patients remarkably improved their oxidative stress condition. It has been proven that antioxidant therapy inhibits airway inflammation and hyperresponsiveness in a mouse model of asthma, suggesting that this may be useful as adjuvant therapy for bronchial asthma (39).

Marked improvement in objective function tests was achieved after three cycles of ozone therapy (Table 4). There were highly significant increments in the mean from FVC and FEV_1 with MAHT (8 mg). However, patients with MAHT (4 mg) did not have statistically significant increments of these function tests, although a trend to elevate these values is observed. Patients with RI (10 mg) had a remarkable increase in FEV_1 but not in FVC. Comparison among the three groups of patients shows that the highest increment for FEV_1 was obtained with MAHT (8 mg) followed by RI (10 mg) and MAHT (4 mg) in agreement with the behavior for the responses of IgE, HLA-DR and antioxidant biomarkers.

Table 4. Effect of ozone therapy on pulmonary function tests before and after three cycles of treatment

	Before		After
		MAHT (4 mg)	
FVC (L)	2.39 ± 0.39		2.63 ± 0.56
FEV_1 (L)	1.56 ± 0.33		1.79 ± 0.53
FEV_1 (%)	49.60 ± 14.68		57.60 ± 20.36
		MAHT (8 mg)	
FVC (L)	2.34 ± 0.28		2.99 ± 0.60**
FEV_1 (L)	1.46 ± 0.20		2.20 ± 0.57***
FEV_1 (%)	46.00 ± 8.10		73.25 ± 19.70***
		RI (10 mg)	
FVC (L)	2.44 ± 0.45		2.59 ± 0.56
FEV_1 (L)	1.59 ± 0.39		1.81 ± 0.52*
FEV_1 (%)	50.53 ± 13.58		59.63 ± 20.46*

*p <0.05; **p <0.02; ***p <0.001.

There was also marked improvement in clinical symptoms. Clinical amelioration achieved in episodic dyspnea, wheezing and medication was reported with the same behavior pattern observed for lung function tests. Therefore, the present data corroborate the beneficial effects of ozone therapy in asthma patients.

In a previous paper we have demonstrated MAHT at 8 mg induces a significant reduction in serum IgE and HLA-DR expression in PBMC, together with a modulation in $CD3^+$, $CD4^+$ and $CD8^+$ lymphocyte subpopulations (40). In our present study, we found similar results for IgE and HLA-DR together with remarkable induction of glutathione antioxidant system and improvement in function and symptom tests using MAHT at an ozone dose of 8 mg and 4 mg and RI at 10 mg. Essentially the agreement in the response pattern for all parameters suggests that ozone therapy modulated the immune system for IgE production along with the induction in antioxidant system. These events allowed the improvement in the asthma state.

The mechanism for immune system modulation is not yet totally proven. We can indirectly assume ozone therapy influenced synthesis and release of cytokines (18,19) in such a way that a change from Th2 to Th1 (IL-2, IFNγ) cytokine pattern occurs, or ozone therapy acted as a Th2 cytokine inhibitor. The change in cytokine pattern could be the reason for the reduction in the release and expression of asthma mediators (IgE, HLA-DR) (41,42). Changes from Th2 to Th1 cytokine pattern have been demonstrated to counteract the airway hyperresponsiveness and bronchial inflammation (43,44). In the presence of IL-4 and IL-13, the B cell undergoes class switching to produce IgE (45), which can induce the release of pro-inflammatory cytokines including the expression of HLA-DR. It is important to remark that the selection of ozone concentration is a fundamental aspect in cytokine release (46,47). Our results are in accordance with this concept because our best results were in the proposed ozone concentration range for cytokine release. Combination of the expressed actions may be the possible mechanism by which ozone therapy has its beneficial effect on asthmatic patients. However, experimental evidence of cytokine type released after ozone therapy application is needed. This could be our next objective.

In summary, our study corroborates the outstanding role of IgE and its direct relationship in the induction of oxidative stress and subsequent bronchial inflammation in allergenic asthma patients. Moreover, we have proven the hypothesis that in these patients systemic ozone therapy reduces IgE level and PBMC HLA-DR expression along with antioxidant system induction. Thus, ozone therapy can be seen as a new therapeutic or adjuvant approach for atopic asthma, thanks to its immunomodulation and oxidative stress regulation properties.

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