Inflammation and Oxidation Biomarkers in Patients with Cystic Fibrosis: The Influence of Azithromycin

Kistik Fibrozisi Olan Hastalarda Enflamasyon ve Oksidasyon Biyobelirteçleri: Azitromisinin Etkisi

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Received: January 19, 2017
Accepted: March 31, 2017

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DOI 10.5152/eurasianjmed.2017.17010

ABSTRACT

Objective: In addition to their antibiotic effect, macrolides appear to modulate the inflammatory response in cystic fibrosis (CF) and could influence oxidative stress. The objective of this study was to assess oxidation biomarkers and levels of inflammation and to determine whether there is an association between these parameters and the intake of macrolides.

Materials and Methods: The subjects included in this cross-sectional study were, on the one hand, clinically stable patients with CF and, on the other, healthy controls. The following serum and plasma inflammatory and oxidative stress biomarkers were measured: interleukin-6 (IL-6), reactive C protein (RCP), tumor necrosis alpha (TNF-α), glutathione peroxidase (GPx), total antioxidant capacity (TAC), catalase (CAT), and superoxide dismutase (SOD), together with markers of lipid peroxidation (8-isoprostanes and thiobarbituric acid reactive substances [TBARS]). Clinical, anthropometric, lung function, radiological, and analytical variables (albumin, prealbumin, vitamins, and zinc) were also recorded.

Results: We studied 36 adults with CF and 41 controls. No differences were observed in age, gender, or anthropometric variables. The patients had significantly higher levels of IL-6, TNF-α, RCP, TBARS, and isoprostanes, and lower levels of SOD than the controls. Twenty-three of the patients were treated with azithromycin, and they had more severe clinical and radiological parameters than those who were not but nevertheless presented significantly lower levels of TNF-α. No differences were observed in the markers of oxidation.

Conclusion: Inflammation and oxidation biomarkers were increased in patients with CF compared with controls. The use of azithromycin was associated with reduced TNF-α levels and did not influence oxidation parameters.

Keywords: Cystic fibrosis, biomarkers, inflammation, oxidation, azithromycin

ÖZ

Amaç: Makrolitlerin, antibiyotik etkilerine ek olarak, kistik fibrolizlerde (KF) enflamatuvar yanıtı değiştirdikleri görülmektedir ve oksidatif stresi etkileyebilirler. Bu çalışmanın amacı enflamasyon düzeylerini ve oksidasyon biyobelirteçlerini değerlendirmek ve bu parametreler ile makrolit alımı arasında bir ilişki olup olmadığını belirlemektir.

Gereç ve Yöntem: Bu kesitsel çalışmaya klinik olarak stabil kistik fibrozis (KF) hastaları ve sağlıklı controller dahil edildi. Şu serum ve plazma enflamatuvar ve oksidatif stres biyobelirteçleri ölçüldü: Lipid peroksidasyon belirteçleri (8-isoprostanler ve TBARs) ile birlikte, interlökin-6 (IL6), C-reaktif protein (CRP), tümör nekroz factor alfa (TNF-α), glutatyon peroksidaz (GPx), total antioksidan kapasite (TAC), katalaz (CAT) ve süperoksit dismutaz (SOD). Aynı zamanda klinik, antropometrik, akciğer fonksiyonu, radyolojik ve analitik değişkenler de (albümin, prealbümin, vitaminler ve çinko) kaydedildi.

Bulgular: Otuz altı yetişkin KF hastası ve kırk bir kontrol grubu hastası ile çalışma yapıldı. Yaş, cinsiyet ve de antropometrik değişkenler açısından farklılık gözlenmedi. Kontrollerle kıyaslandığında, KF hastalarında IL-6, TNF-α, RCP, TBARS ve izoprostan düzeyleri önemli ölçüde daha yüksekti ve SOD düzeyleri daha düşüktü. Hastaların 23'üne azitromisin tedavisi verildi ve bu tedaviyi almayan hastalara göre daha ağır klinik ve radyolojik parametreler sergilediler, ancak TNF-α düzeyleri anlamlı ölçüde daha düşüktü. Oksidasyon belirteçlerinde farklılık gözlenmedi.

Sonuç: Enflamasyon ve oksidasyon biyobelirteçleri KF hastalarında kontrollere göre yüksekti. Azitromisin kullanımı düşük TNF-α düzeyleri ile ilişkili bulundu ve oksidasyon parametrelerini etkilemedi.

Anahtar Kelimeler: Kistik fibrozis, biyobelirteçler, enflamasyon, oksidasyon, azitromisin

Introduction

Patients with cystic fibrosis (CF) endure a progressive pulmonary pathology that is governed by a chronic, exaggerated inflammatory response. As it happens in other respiratory diseases such as chronic obstructive pulmonary disease (COPD), progression of the disease is characterized by chronic, unopposed, neutrophil-dominated inflammation, which is associated with reduced lung function [1-3]. The damaged airway epithelium impairs mucociliary clearance and provokes the retention of secretions, which in turn attracts bacterial colonization and predisposes the patient to repeated infections and bronchiectasis. Therefore, the ultimate goal in treating the disease is to break this vicious cycle and to prevent or retard progressive inflammatory lung damage [4]. The benefits of macrolide antibiotics in the treatment of CF are well known [5]. Azithromycin (AZM) presents anti-inflammatory, antimicrobial, and immunomodulatory properties, which contribute to the improvement of clinical parameters, including delayed lung function decline, time to acute pulmonary exacerbation, and the need for antimicrobial treatment [6-10].

The mechanisms of this effect are not fully understood, but macrolides have been shown to affect many pathways of the inflammatory process, such as the production of pro-inflammatory cytokines, the oxidative burst in phagocytes, and the migration of neutrophils [11].

Oxidative stress seems to play an important role in the pathophysiology of CF. Many factors may contribute to increased oxidative stress in CF, as the disease combines impaired antioxidant protection with the increased production of reactive oxygen species. Oxidative stress markers are often raised despite routine supplementation with liposoluble antioxidant vitamins, such as vitamin E or carotenoids [12]. Oxidative stress has been shown to produce lung damage in CF patients at an early age [13, 14].

The objective of this study was to assess biomarkers of inflammation and oxidation in adult patients with CF and to determine whether there is an association between these biomarkers and treatment with macrolides under conditions of routine clinical practice.

Materials and Methods

Study design and patients

We describe a cross-sectional study. Thirty-six patients with a diagnosis of CF were included. All patients were aged >16 years and were periodically monitored in our adult CF unit [15, 16]. A control group comprised 41 healthy subjects who matched with the patients with respect to nutritional status, age, and sex. All patients signed an informed consent agreement to participate in this study, which was approved by the ethics and research committee of our university hospital.

Patients were recruited in a stable phase and with no evidence of any exacerbations for at least 90 days. Patients were excluded if they were taking dietary supplements with omega-3 fatty acids, had not completed puberty, were on the transplant waiting list, or had received a transplant.

Patients with CF with frequent exacerbations, chronic bronchial colonization by pathogenic microorganisms, and/or FEV_1 <50% were given AZM 500 mg per day, three times per week, for at least six months, as an immunomodulatory treatment. Before the treatment, an electrocardiogram was performed on these patients, and the AZM was not given in case of long QT syndrome.

Clinical, lung function, radiological, and analytical variables

The anthropometric measurements taken included height, using a stadiometer (Holtain limited, Crymych, UK), and weight, using a SECA 665 scale (SECA, Hamburg , Germany), to calculate body mass index (BMI). Skinfold thickness (subscapular, tricipital, dominant bicipital, and abdominal) measurements were determined using a constant pressure lipocalibrator (Holtain limited, Crymych, UK). Three measurements were completed for each site, and the mean value. The fat-free mass was estimated from the equations of Durnin et al. [17] and Siri [18] and divided by the square of the height to determine the fat-free mass index (FFMI). Hand dynamometry was performed with a Jamar hydraulic dynamometer (Asimow Engineering Co., USA) obtaining three measurements on the dominant hand, and the values were averaged.

A prospective dietary questionnaire was administered to all the patients [19].

Simple and forced spirometry (Jaeger Oxycon Pro®, Erich Jaeger, Germany) was performed following international guidelines and expressed as a percentage of the theoretical value in a reference population [20].

A full clinical history, from diagnosis through to study participation, was recorded in a database: information was collected at each visit (every two to three months) on clinical and demographic variables, including respiratory exacerbations, using the Spanish Society of Pulmonary and Thoracic Surgery criteria, and a sputum sample was collected for microbiological study [15]. Initial colonization by microorganisms was analyzed, taking into account their appearance in sputum (at least three positive occurrences), regardless of their persistence at the time of the study. Measurement of the mean amount of sputum produced daily (in milliliters) was evaluated following the protocol of Martínez-García et al [21].

We used the Bhalla scoring test to assess the structural damage with high resolution computed tomography (lower values indicate greater damage) [22].

Blood samples were collected after a 12-hour fast. Plasma and serum were aliquoted and stored until analysis at -70°C in the Hospital-IBIMA Biobank, which forms part of the biobank of the public health system of Andalusia (BSSPA) and of the Spanish National Biobank Platform (PT13/0010/0006).

Fat-soluble vitamins E and A were analyzed by high-performance liquid chromatography (Agilent 1200 of Bio-Rad). Vitamin D was analyzed by electrochemiluminescence immunoassay (Modular E-170, Roche Diagnostics). Zinc was measured by atomic absorption spectrophotometry (AAnalyst 200, Perkin Elmer, USA).

Serum and plasma oxidative stress biomarkers

Glutathione peroxidase (GPx) activity, total antioxidant capacity (TAC), catalase (CAT) activity, and superoxide dismutase (SOD) activity were measured in the plasma using commercial kits (Cayman Chemical, Ann Arbor, USA). 8-iso-prostaglandin F2α (8-iso-PF2α) was analyzed by a competitive enzyme-linked immunoassay (Cell Biolabs Inc.; USA). Thiobarbituric acid reactive substances (TBARS) were determined by spectrophotometry [23].

Statistical analysis

Data were expressed as mean with standard deviation (SD) for quantitative variables and as percentages for categorical variables. The Kolmogorov-Smirnov test was used to analyze the distribution of variables. Quantitative variables for two groups were compared with the Student t-test or the Mann-Whitney test, when necessary. Qualitative variables were compared with the chi-square test or Fisher's test as appropriate. P values <0.05 indicated statistical significance. R statistical software was used for data analysis [24].

Results

Cases versus controls

The study population consisted of 36 patients with CF and 41 controls. The relevant characteristics of both groups are summarized in Table 1. The mean intake (kcal/kg) and the percentages of carbohydrates and of polyunsaturated fats were significantly higher in the CF subjects, but the values for monounsaturated fats (percentage) were lower.

Biomarkers of inflammation and oxidation

Significantly higher levels of interleukin-6 (Il-6), tumor necrosis alpha (TNF-α), reactive C protein (RCP), CAT activity, TBARS, and isoprostanes were observed in the CF group, together with lower levels of SOD, vitamin D, and vitamin A.

The influence of azithromycin

The group treated with AZM was composed of 23 patients (Table 2) who started treatment at a mean age of 25.2±9.2 (16-58 years) and had been taking AZM for an average of 38±22 months before the clinical analysis was performed. No significant differences were observed in anthropometric parameters, dynamometry, or the results of the dietary survey.

The group that was treated with macrolides had a significantly higher percentage of F508del homozygotes, meconium ileus, and chronic colonization in comparison with the patients who received no treatment. Statistically significant differences (with higher values in the treated group) were also obtained for the sweat test, the number of exacerbations (whether mild or severe) and the Bhalla test (with lower scores in the treated group) and spirometric parameters. Levels of vitamin D were significantly lower in the CF subjects treated with macrolides. Regarding the parameters of oxidation and inflammation, the only significant differences found were in the levels of TNF-α (lower in the group that received AZM).

Table 1. Clinical parameters, characteristics of the dietary survey, and analytic parameters in cases and controls

		CF (36)	Controls (41)
Age	(m±sd)	27.2±8.9	29.0±9.6
Sex	n (%)		
Male		(18) 56.3%	(14) 43.8%
Female		(18) 40%	(27) 60%
Weight (kg)	(m±sd)	61.1±10.8	64.0±10.8
Height (m)	(m±sd)	164.6±8.0	167.5±9.8
BMI (kg/m²)	(m±sd)	22.5±3.2	22.7±2.3
DIETARY SURVEY			
Energy consumption/weight (kcal/kg)	(m±sd)	45.38±14.09***	31.89±8.03
Carbohydrates (% kcal of the total)	% (m±sd)	45.2±7.4**	41.5±4.6
Proteins (% kcal of the total)	% (m±sd)	15.4±3.4	16.2±2.5
Lipids (% kcal of the total)	% (m±sd)	38.7±6.2	40.9±4.5
-Saturated fats	% (m±sd)	29.2±7.0	28.3±5.6
-Polyunsaturated fats	% (m±sd)	16.4±5.9*	13.3±3.8
-Monounsaturated fats	% (m±sd)	54.3±7.4**	58.5±5.5
-W6 fatty acids	% (m±sd)	14.3±5.4	11.7±3.8
-W3 fatty acids	% (m±sd)	2.06±2.6	1.5±0.5
-W6 fatty acids / W3 fatty acids	% (m±sd)	9.7±5.6	8.2±3.3
OXIDATION			
Total antioxidant capacity (mmol TE)	(m±sd)	2.02±1.08	2.35±0.93
Catalase activity (nmol/min/mL)	(m±sd)	119.43±34.07***	98.62±36.65
Superoxide dismutase (SOD) activity (UI/mL)	(m±sd)	7.30±2.12***	10.40±3.67
Glutathione peroxidase (GPx) activity (nmol/min/mL)	(m±sd)	88.60±7.96	87.13±8.32
TBARS (lipid peroxidation) (micromol/L)	(m±sd)	0.14±0.046**	0.11±0.031
Isoprostanes (8-iso-PG2α) (pg/mL)	(m±sd)	111.68±34.55*	88.08±29.17
INFLAMMATION			
IL-6 (pg/mL)	(m±sd)	12.35±20.13 **	3.11±2.83
TNF-α (pg/mL)	(m±sd)	3.61±1.60***	2.60±0.45
RCP (mg/L)	(m±sd)	11.75±13.63***	1.30±4.18
ANTIOXIDANTS			
Vitamin D (mcg/dL)	(m±sd)	24.8±11.9*	30.9±7.5
Vitamin A (mcg/dL)	(m±sd)	30.1±9.2***	39.6±9.6
Vitamin E: cholesterol (mg/g)	(m±sd)	6.4±1.6	5.9±0.5
Zinc (mcg/dL)	(m±sd)	85.9±13.5	80.0±4.9

CF: cystic fibrosis; BMI: Body Mass Index; TBARS: thiobarbituric acid reactive substances; IL-6: interleukin-6; TNF-α: tumor necrosis factor alpha; RCP: reactive C protein; mmol TE: millimolar Trolox equivalents

*p<0.05; **p<0.01; ***p<0.001. Significant differences between patients with cystic fibrosis and controls

Discussion

This study was performed to evaluate the influence of AZM on the parameters of inflammation and oxidation in clinically stable patients with CF. The results show that those who received AZM had lower levels of TNF-α than those who do not receive it, despite having a more severe form of the disease.

As expected (and in accordance with previous studies in this field), the patients had significantly higher levels of RCP, IL-6, and TNF-α than the controls, despite being in a clinically stable state [25, 26]. These data support the hypothesis that there is a state of subclinical inflammation associated with CF that can be measured systemically [27, 28]. Other studies, also of clinically stable patients, have reported associations and correlations between the levels of pro-inflammatory cytokines (RCP, IL-6, and TNF-α) and the severity of respiratory disease, determined using various parameters, both clinical (exacerbations, bronchorrhea, colonization by *Pseudomonas aeruginosa* [PA], desaturation with exercise) spirometric (FEV_1 %) and radiological (Bhalla score) [29]. This supports the idea that serum inflammatory mediators could be good markers of the severity of the disease and that these would correlate with the prognosis of subjects with CF.

Table 2. Clinical parameters, characteristics of the dietary survey, and analytic parameters in patients with and without azithromycin

	Azithromycin (n=23)	No treatment (n=13)	Significance
Age (m±sd)	28.4±9.5	25.2±7.5	NS
Sex n (%)			NS
Male	(11) 47.8%	(7) 53.8%	
Female	(12) 52.2%	(6) 46.2%	
F508del	7.7%	39.1%	0.034
Sweat test (mmol/L) (m±sd)	114.4±12.2	104.5±13.6	0.026
BMI (kg/m^2)	22.1±2.8	23.1±3.9	NS
Fat-free mass index (kg/m^2)	17.2±2.3	17.6±2.1	NS
Dynamometry (kg)	26.9±12.2	29.0±13.3	NS
Diabetes n (%)			NS
No	(16) 69.6%	(8) 61.5%	
Yes	(7) 30.4%	(5) 38.5%	
Exocrine pancreatic insufficiency			NS
No	(7) 30.4%	(5) 38.5%	
Yes	(16) 69.6%	(8) 61.5%	
Meconium ileus n (%)			0.044
No	(17) 73.9%	(13) 100%	
Yes	(6) 26.1%	(0) 0%	
Respiratory insufficiency with effort			NS
No	(1) 7.7%	(12) 92.3%	
Yes	(6) 26.1%	(28) 77.8%	
Colonizations	100%	76.9%	0.016
Exacerbations	3.1±2.1	1.2±1.2	0.003
Mild exacerbations	1.2±1.1	2.4±1.6	0.016
Severe exacerbations	0.7±1.3	0.15±1.4	0.05
Bhalla	14.1±3.6	17.0±2.7	0.022
FEV1 (%)	47.1±21.3%	77.1±20.9%	0.000
FVC (%)	60.1±21.8%	82.9±17.1%	0.003
Albumin (mg/dL)	3.7±0.4	3.9±0.3	NS
Prealbumin (mg/dL)	20.2±5.5	21.9±5.9	NS
Vitamin A (mcg/dL)	29.5±8.8	31.2±10.3	NS
Vitamin D (mcg/dL)	21.8±9.7	30.9±14.0	0.035
Vitamin E (mcg/dL)	833.0±190.1	937.4±370.8	NS
Vitamin E/Cholesterol (mg/g)	7.1±1.1	7.0±2.3	NS
Zinc (mcg/dL)	84.3±12.3	88.5±16.62	NS
OXIDATION			
Total antioxidant capacity (mM)	2.0±1.2	2.1±0.8	NS
Catalase activity (nmol/min/mL)	116.1±33.7	125.2±35	NS
Superoxide dismutase (SOD) activity (UI/mL)	6.8±2.0	8.1±1.9	NS (0.06)
Glutathione peroxidase (GPx) activity (nmol/min/mL)	90.3±8.3	85.6±6.4	NS
TBARS (lipid peroxidation micromol/L)	0.147±0.04	0.137±0.05	NS
Isoprostanes (8-iso-PG2α) (pg/mL)	112.8±32.9	109.6±38.6	NS
INFLAMMATION			
IL-6 (pg/mL)	13.9±24	9.7±10	NS
TNF-α (pg/mL)	3.1±0.6	4.5±2	0.010
RCP (mg/L)	13.9±14	8.2±11.5	NS

BMI: Body Mass Index; FEV1: forced expiratory volume in one second; FVC: forced vital capacity; TBARS: thiobarbituric acid reactive substances; IL-6: interleukin-6; TNF-α: tumor necrosis factor alpha; RCP: reactive C protein

As expected, the group treated with AZM presented greater disease severity, in terms of clinical, radiological, and laboratory analysis results (higher rate of exacerbations, both mild and severe, poorer spirometric values, lower Bhalla score, and lower levels of vitamin D); moreover, these patients were carriers of more severe mutations (higher percentage of F508del homozygotes) and had a significantly higher percentage of chronic colonization and of meconium ileus. This greater severity would lead us to expect, at least theoretically, higher levels of cytokines and oxidative stress among clinically stable patients than among those who did not receive this medication. However, in practice we recorded lower levels of TNF-α and observed no significant differences in the other evaluated parameters (IL-6 and RCP) or in the plasma markers of oxidation. This might be because macrolides modulate the inflammatory response and, possibly, oxidative stress. AZM reduces respiratory exacerbations in CF and in non-CF bronchiectasis and the need for oral antibiotics against PA [30]. AZM may also reduce the secretion of TNF-α in the epithelial cells of the airway [31]. However, conflicting views have been published in this respect, and some in vitro studies have found that AZM does not reduce the levels of TNF-α in CF [32].

We found, in agreement with other studies, that serum or plasma markers of oxidation differed significantly between patients and controls (higher levels of CAT activity, higher markers of lipid peroxidation, and lower levels of SOD in the former) [12]. CF often provokes an imbalance of oxidants and antioxidants (especially in patients with exocrine pancreatic insufficiency).

However, our results did not reveal any differences with respect to the parameters of oxidative stress (TAC, CAT activity, SOD activity, GPx activity, TBARS and isoprostanes) between the patients who were taking AZM and those who were not. In other studies that have reported similar results in patients with non-CF bronchiectasis and receiving long-term AZM treatment, there were no differences in these oxidative parameters, despite the reduced exacerbations, improved symptoms, and enhanced quality of life [33]. This may be because AZM has little or no effect on oxidative stress, with the beneficial clinical effects being due to other mechanisms of action [7].

Among our sample, levels of vitamin D were lower in the CF patients than in the controls, and also in those taking macrolides than those who were not [34]. Vitamin D has been shown

to suppress in vivo and in vitro the production of inflammatory cytokines, such as TNF-α, IL-6, and IL-8, and to increase the production of the antimicrobial peptide, IL-37, from CF respiratory epithelial cells. Moreover, for CF patients hospitalized with a pulmonary exacerbation, a bolus dose of vitamin D is associated with reduced levels of the inflammatory cytokines TNF-α and IL-6. On the other hand, serum 25(OH)D levels are significantly associated with pulmonary dysfunction in CF patients [35].

Subjects with CF, non-CF bronchiectasis, or COPD present elevated plasma levels of TNF-α, which are associated with reduced lean mass, increased muscle proteolysis, increased respiratory exacerbations and poorer lung function, even in clinically stable patients [36]. In vivo and in vitro studies suggest that IL-6 and TNF-α may play a regulatory role in the maintenance of skeletal muscle mass [37]. However, our study revealed no differences in the FFMI between CF patients and controls or between those who took AZM versus those who did not. This latter fact could be because the AZM used in long-term treatment seems to improve the nutritional status of CF subjects, improving BMI by increasing body weight [38].

This study presents the following strengths: we measured different biomarkers of oxidation and inflammation in clinically stable patients; the patients were compared with a control group; and the study was conducted under conditions of routine clinical practice. However, it is not without limitations. On the one hand, the transversal nature of the study limits the possibilities of inferring causality. Furthermore, the sample was relatively small, and although a large number of biomarkers were used. We do not know which are the best markers of inflammation and oxidation for CF patients; thus, the choice of different markers might have yielded different results.

Inflammation and oxidation biomarkers were increased in patients with CF compared with controls. AZM could modulate the inflammatory response in patients with CF and is associated with decreased levels of systemic TNF-α, while parameters of oxidation remain unaffected. This may have a beneficial effect on various clinical parameters. Further studies (prospective and with a sufficient number of patients) are needed to confirm these findings.

Ethics Committee Approval: Ethics committee approval was received for this study from Hospital Regional University of Málaga (Decision Date: 13.05.2007/Decision No: 7).

Informed Consent: Written informed consent was obtained from patients who participated in this study.

Peer-review: Externally peer-reviewed.

Author contributions: Concept - G.O., C.O.; Design - G.O., C.O.; Supervision - G.O., C.O.; Resource - G.O., C.O.; Materials - C.O., A.P., A.D., V.C., E.G.F., E.R.M., N.P., E.D., A.C., G.O.; Data Collection and/or Processing - C.O., A.P., A.D., V.C., E.G.F., E.R.M., N.P., E.D., A.C., G.O.; Analysis and /or Interpretation - G.O., C.O.; Literature Search - G.O., C.O., A.P.; Writing - G.O., C.O.; Critical Reviews - C.O., A.P., A.D., V.C., E.G.F., E.R.M., N.P., E.D., A.C., G.O.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: This study was supported by a grant from the Consejería de Salud de la Junta de Andalucía (Andalucía-Spain) PI-0525/2009 and CIBERDEM (CIBER of Diabetes and Associated Metabolic Diseases).

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