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Artigo Original

Oxidative stress and inflammatory parameters in adult patients with CD

Estresse oxidativo e parâmetros inflamatórios em pacientes adultos com doença celíaca

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Keywords

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Celiac disease is a chronic systemic inflammatory condition caused by an inappropriate immune response to gluten of wheat, rye and barley, with a prevalence of about 1: 100 in the Caucasian population when occurs inflammatory response and seems to involve high levels of interleukins. Objective: Determine the presence of oxidative stress and inflammation in the gut CD patients Methods: Transversal study including patients undergoing upper gastrointestinal (GI) endoscopy was performed. The study population consisted 24 cases and 26 controls. The duodenal levels of protein carbonyls, thiobarbituric acid reactive species (TBARS), as well as catalase (CAT), superoxide dismutase (SOD) activities were measured. Gut levels of interleukin (IL) 6, 8 and 10 were also determined. The Marsh classification was recorded and used as a parameter of severity. Results: Both IL-6 and IL-10, but not IL8, were increased in CD patients when compared to healthy individuals. Oxidative damage parameters were increased while antioxidant defenses were decreased in our sample. Both IL6 levels and SOD activity were related to Marsh score. Conclusions: Different markers of inflammation and oxidative stress are altered in the gut of CD patients, and some of them are related severity.

Palavras-chave

Doença celíaca
Gastroenterologia
Parâmetros inflamatórios
Estresse oxidativo
Nutrição

Doença celíaca (DC) é uma condição inflamatória crônica sistêmica causada por uma resposta imunitária inapropriada ao glúten de trigo, centeio e cevada, com uma prevalência de 1:100 na população caucasiana, onde ocorre uma resposta inflamatória e parece envolver elevados níveis de interleucinas. Objetivo: Determinar a presença de estresse oxidativo e inflamação no intestino de pacientes com DC. Método: Para isso realizamos um estudo transversal que incluiu pacientes submetidos a endoscopia gastrointestinal superior. A população do estudo consistiu de 24 casos e 26 controles. Os níveis duodenais de carbonilação proteica, espécies reativas ao ácido tiobarbitúrico (TBARS), bem como a atividade da catalase (CAT) e da superóxido dismutase (SOD) foram mensuradas. Níveis de interleucina (IL), 6, 8 e 10 também foram mensuradas no intestino desses pacientes. A classificação da escala Marsh foi registrada e utilizada como um parâmetro da gravidade da doença. Resultados: Tanto IL-6 e IL-10, mas não IL8, foram aumentados em pacientes com DC quando comparados a indivíduos saudáveis. Parâmetros de dano oxidativo foram aumentados, enquanto as defesas antioxidantes foram diminuídas em nossa amostra. Níveis de IL6 e atividade da SOD foram relacionados a pontuação Marsh. Conclusão: Diferentes marcadores de inflamação e estresse oxidativo são alteradas no intestino de pacientes com DC, e alguns deles estão relacionados com a gravidade da doença

INTRODUCTION

Celiac disease (CD) is an immune-mediated systemic disorder elicited by gluten of wheat, rye and barley and related prolamines in genetically susceptible individuals and is characterized by the presence of a variable combination of gluten-dependent clinical manifestations, CD-specific antibodies, HLA-DQ2 or HLA-DQ8 haplotypes, and enteropathy[1]. The disease is common with a prevalence of about 1: 100 in the Caucasian population. In Brazil, Muniz et

al.[2] detected that 49.1% of blood donor candidates from São Paulo state have genetic predisposition to develop CD. In a 2012 Brazilian study involving 452 adult individuals from different regions of the country, the prevalence of DQ2 and/or DQ8 was similar to Muniz et al.[2] results. In Brazil, the prevalence of genetic markers for CD has been as high as that found in Europe and China.

The histopathology of CD is characterized by villous blunting, crypt hyperplasia and increased number of intraepithelial lymphocytes. In treated patients gluten challenge induces an accumulation of CD14(+) CD11c(+) dendritic cells[3] and this could induce activation of lymphocytes[4].

This unique inflammatory response seems to involve also the innate immune system since CD patients showed high levels of Toll Like Receptor (TLR) 4 expression and interleukins (IL1, IL6, IL8, and IL17)[5]. This kind of response could suggest that microbiota-associated factors may be important in the development of the disease[6]. Despite of this a more detailed description of the relationship between cytokines, chemokines and the cellular consequences of inflammation (such as oxidative damage) and DC severity is needed.

Within this perspective, this research aims to determine the presence of oxidative stress and inflammation in the gut of patients with CD, comparing these markers (oxidative stress and inflammation) with histopathological markers of severity.

PATIENTS AND METHODS

This study was conducted in a city of Southern of Santa Catarina from February to June 2014 and was approved by the local Ethics Committee (529 014/2014). All patients signed the Free and Informed Consent (FIC).

PATIENTS

It was a transversal study that included patients undergoing upper gastrointestinal (GI) endoscopy in a tertiary hospital from February to June 2014. Adult patients (> 18 years old) were included if they had complaints suggestive of celiac disease (diarrhea or constipation), and the diagnosis was confirmed afterwards by histologic and serologic evaluation (antitransglutaminase antibodies [TGA], and antiendomysium antibodies [EMA])[7]as suggested by the American College of Gastroenterology[8]. In this way, patients were not being treated at the moment of sampling (the treatment consists of a gluten-free diet). In addition, patients with normal histology were included, and they had undergone an upper GI endoscopy as part of the routine diagnostic workup (serologic evaluation was performed in patients too).

Exclusion criteria: immunosuppression, infectious diseases in the last 30 days and cancer.

Duodenal samples (one sample per patient) were collected during upper GI endoscopy in the same duodenal area taken for histopathological examination. During the histopathological analyses the number of lymphocytes was quantified as well as the Marsh classification recorded. Relevant clinical information was collected directly from the patients or reviewing medical charts. Patients did not follow the gluten-free diet before exams for this research.

METHODS

Oxidative damage to proteins was assessed by determining the carbonyl groups of the sample, based on the reaction with dinitrophenylhydrazine (DNPH). Briefly, proteins were precipitated by addition of 20% trichloroacetic acid, dissolved in guanidine and mixed with DNPH. Protein carbonyls were determined by the absorbance at 370nm and expressed as nmol/mg protein[9]. As an evidence of lipid peroxidation it was measured thiobarbituric acid reactive species (TBARS) levels in a heated acidic reaction. Briefly, samples were mixed with 10% trichloroacetic acid and of thiobarbituric acid. The solution was boiled for 15 minutes, and the amount of TBARS determined by absorbance at 535 nm[10]. TBARS levels were expressed as MDA equivalents (nmol/mg protein).

Superoxide dismutase (SOD) activity was measured by the inhibition of autoxidation adrenaline followed spectrophotometrically at 420 nm. A calibration curve was made using as a standard purified SOD in order to calculate the specific activity of SOD present in the samples. A 50% inhibition of the autoxidation was defined as one unit of SOD and the specific activity was represented as units per mg protein[11]. The activity of catalase (CAT) was determined by the rate of H_2O_2 clearance at 240 nm. A unit of CAT is defined as one mole of hydrogen peroxide consumed per minute and the specific activity was reported as units per mg protein[12].

As inflammatory parameters, two cytokines (IL6 and IL10) and one chemokine (IL8) were measured by ELISA kits as recommended by the manufacturer (Peprotech, FUNPEC BRAZIL - SP). The unit of measure was pg/mg protein.

After collecting data, it was designed a database on IBM software Statistical Package for Social Sciences (SPSS) version 21.0. Quantitative variables were expressed as mean and standard deviation and qualitative by frequency and percentages. An association between qualitative variables and the presence of DC was investigated by chi-square and Fisher exact tests. The magnitude of the association was estimated by calculating the odds ratio (OR). The difference between quantitative variables and the presence of DC was investigated by the Mann-Whitney U test, preceded by the Shapiro-Wilk test. Correlation between quantitative variables was determined by Spearman test. The relation of the biomarkers with the Marsh classification was determined by Kruskal-Wallis test. Statistical tests were performed with a significance level $\alpha = 0.05$ and 95% confidence.

RESULTS

The study population consisted of 50 patients, of whom 24 had a diagnosis of CD. CD patients are significantly older when compared to healthy individuals (Table 1). When it was analyzed the presence of GI symptoms, only the presence of diarrhea was significantly more prevalent in CD patients (Table 1).

Table 1. Clinical characteristics of patients

Variable	Celiac disease		OR	IC 95%	P value
	Yes (n=24)	No (n=26)			
Age (years)	37.50±13.54	31.27±11.17	-	-	0.019
Sex					
Female	21(87.5)	22(84.6)	0.786	0.157-3.938	1.00
Race					
Caucasian	21(87.5)	24(92.3)	0.583	0.089-3.833	0.661
Diarrhea					
Yes	18(75.0)	12(46.2)	3.500	1.051-11.660	0.038
Pain and abdominal distention					
Yes	20(83.3)	23(88.5)	0.652	0.130-3.271	0.697
Flatulence					
Yes	14(58.3)	13(50.0)	1.400	0.458-4.281	0.555
Anemia					
Yes	6(25.0)	6(23.1)	1.111	0.303-4.071	0.874
Stomatitis					
Yes	10 (41.7)	10(38.5)	1.143	0.368-3.547	0.817

Since it is believed that inflammation has a central role in the pathogenesis of CD it was measured the duodenal levels of IL6, IL10 and IL8. Both IL-6 and IL-10 were increased in CD patients when compared to healthy individuals (Table 2). This was not true IL-8 level.

One of the major consequences of inflammation is oxidative stress, thus it was also determined oxidative stress levels in our sample. Both TBARS and protein carbonyls levels were increased in the duodenum of CD patients (Table 2). Oxidative damage could also be secondary to an imbalance in antioxidant defenses. In this way, the activity of both CAT and SOD were decreased in patients with CD (Table 2).

Table 2. Oxidative stress and inflammatory parameters in Celiac Disease patients

Variable	Celiac disease		p value
	Yes (n = 24)	No (n=26)	
Carbonyl (nmol/mg protein)	0.021±0.009	0.015±0.003	0.002
TBARS (nmol/mg protein)	0.007±0.001	0.003±0.001	<0.001
SOD (U/mg protein)	1.464±0.188	5.239±1.410	<0.001
CAT (U/mg protein)	0.517±0.234	1.013±0.345	<0.001
IL10 (pg/mg protein)	7.175±1.740	4.729±1.701	0.002
IL6 (pg/mg protein)	16.989±7.342	9.654±3.831	0.002
IL8 (pg/mg protein)	7.438±2.294	5.882±1.729	0.15

Carbonyl - protein carbonyls; SOD – superoxide dismutase; TBARS – thiobarbituric acid reactive species; CAT – catalase ; IL – interleukin

It was further explored if there is any correlation between histopathological markers of CD severity and inflammatory or oxidative stress biomarkers. There was not any significant correlation between the numbers of lymphocytes in duodenal biopsies and either inflammatory or oxidative parameters (data not shown). In contrast, patients with Marsh score 1 presented significantly higher SOD activity and lower IL6 levels when compared to patients with Marsh score 2 and 3 (Figure 1). In our sample there was no patient with Marsh score 0 or 4 (CD was considered only with Marsh score 2 and 3 in this study according with Modified Marsh Classification of histologic findings in celiac disease (Oberhuber). There was no significant variation between either catalase, IL10 or IL8 when comparing different Marshal scores.

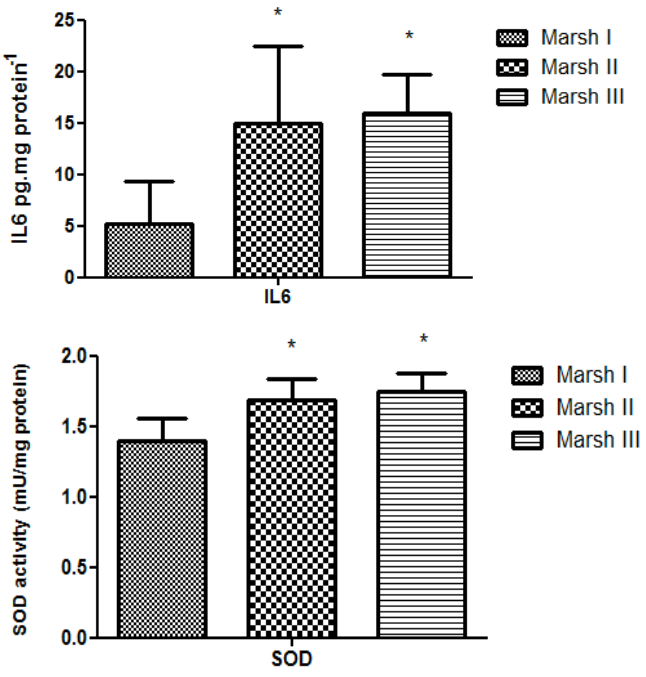

Figure 1 – Histopathological severity index and its relation with inflammatory and

oxidative stress markers. * Significantly different from Marsh I, p <0.05

It was noticed that both IL-6 and IL-10, but not IL8, were increased in CD patients when compared to healthy individuals. Oxidative damage parameters were increased while antioxidant defenses were decreased in our sample. Both IL6 levels and SOD activity were related to Marsh score.

DISCUSSION

We have here demonstrated that both oxidative stress and inflammatory parameters are associated with CD, and both SOD and IL6 levels are related to Marsh score.

IL-6 is a cytokine secreted by T cells and macrophages to stimulate the immune response mainly during infection. It is well known that both Th1 and Th17 lymphocytes are able to secrete IL-6, thus it is expected that during CD development there is an increase in gut IL-6 levels. As we demonstrated here, Eiró and cols[5] showed that both children and adult CD patients have increased gut levels of several Th1/Th17 related cytokines, including IL6. In treated CD patients a challenge with gliadin induces the expression of IL6 in gut mucosa[13] Despite of this Medrano and cols.[14] did not find significant correlations between Th17-related genes polymorphisms (including IL6) in CD patients when compared to healthy individuals.

IL-10 was increased in CD patients when compared to healthy individuals. IL-10 is a cytokine with anti-inflammatory properties and plays an important role in inflammation, regulating the immune system[15]. It is secreted by Th2 and T regulatory lymphocytes. CD was originally described as a purely Th1 disease. Probably it involves Th17 and T regulatory lymphocytes as well[16-17]. It is proposed that new therapies to CD involves the suppression of Th1 / Th17 activation by a cross-regulation of the immunological response by concurrent Th2 activation or IL10 production[18]. Thus, the increase in IL10 levels could be related to an adaptive response to the Th1/Th17 response naturally associated with the development of CD. This is supported by the finding that potential CD patients (defined as subjects who do not have, and have never had, a jejunal biopsy consistent with clear CD, and yet have immunological abnormalities similar to those found in celiac patients)[19] show activation of regulatory mechanisms (such as increasing IL10 mRNA levels) and is believed that this is a mechanism to prevent the progression toward a mucosal damage[20].

IL-8 is produced by macrophages and other cell types such as epithelial cells, airway smooth muscle cells and endothelial cells. Its main function is induce the chemotaxis of neutrophils. The role for IL8 during CD development is not well understood. IL8 is up-regulated, for example, in ulcerative colitis, and the remission of the disease is associated with a normalization of IL8 gene expression[21]. In literature none study determined IL8 levels in the gut of CD patients. One study found an increase in IL8 gene expression in the gut of CD patients[4], but we are not able to demonstrate any significant difference in protein levels between CD and control patients. IL8 is mainly produced by the innate immune system, and despite some evidence for a role of this response in CD, it is classically related to the activation in the acquired immune system. Certain gliadin peptides are able to induce an innate immune response probably through the activation of TLRs. TLR activation could then increase the secretion of several cytokines, including IL6 and IL8. A better understanding on the modulation of TLR responses is needed to explain these non-concordant findings[22].

In addition to immunogenic effects, gliadin may directly affect intestinal cell structure and function. One of these mechanisms seems to be related to oxidative stress. Gliadin exposure induces an oxidative imbalance, and some markers of oxidative stress, such as 4-hydroxy-2-nonenal and an increase in the oxidation (GSSG)/reduced (GSH) glutathione ratio have been demonstrated in vitro[23]. Duodenal biopsies of CD patients also demonstrated markers of oxidative damage, as shown here demonstrated[24-25]. Differently from what we found, in children SOD activity seems to be increased in the gut of CD patients, CAT did not change and there was a decrease in the glutathione-related antioxidant defenses[25]. SOD is an enzyme with antioxidant properties. In this study patients with scores 2 and 3 showed higher activity of SOD, this can be explained as a mechanism of protection against damage caused by celiac disease. Thus, it seems that oxidative damage could be related to antioxidant defenses alterations, opening the perspective of an antioxidant-based treatment for CD[26].

Some suggestions must be taken into consideration when interpreting our results. It were included newly diagnosed with CD patients, thus we are were not able to establish the stage of disease development at the moment of gut biopsies. Due to the transversal design of the study it were not able to determine if CD treatment would interfere in the analyzed parameters and if they could predict disease outcomes. The longitudinal observation of CD patients is of pivotal importance to understand better the role of inflammation and oxidative stress in disease progression.

CONCLUSIONS

Different markers of inflammation and oxidative stress are altered in the gut of CD patients, and some of them are related to disease severity. Inflammatory markers, most often associated with Th1/Th17 and Th2 balance, appear to be relevant to CD development, as well as oxidative damage and antioxidant defense imbalance.

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