FOLIA MEDICA CRACOVIENSIA
Vol. LVIII, 2, 2018: 119–130
PL ISSN 0015-5616
DOI: 10.24425/fmc.2018.124663

Evaluation of plasma concentrations of selected antioxidant parameters in patients with active Crohn's disease

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Abstract: Oxidative stress (OxS) has been implicated in the pathogenesis of Crohn's disease (CD). The aim of this study was to examine whether nonenzymatic antioxidants are associated with active CD, by using the FRAP and GSH assay in plasma. Additionally, we measured bilirubin and albumin levels as two individual components of the plasma antioxidant system.

A total of 55 patients with established CD, 30 with active CD and 25 with inactive disease, and 25 healthy individuals were prospectively enrolled in this study. We evaluated CD activity index, BMI and blood morphology, platelet count, serum CRP level, and bochemical parameters of OxS: ferric reducing ability of plasma (FRAP), reduced glutathione (GSH) in plasma and bilirubin and albumin levels in serum.

Plasma FRAP and GSH concentrations were decreased in both CD groups compared to controls and negatively correlated with CDAI values (FRAP: $r = -0.572$, $p = 0.003$; GSH: $r = -0.761$, $p = 0.001$), CRP and platelet count. Bilirubin and albumin levels were lower in the serum of active CD patients than inactive CD patients and controls and negatively correlated with the CD activity index ($r = -0328$, $p = 0.036$, $r = -0.518$, $p = 0.002$) and CRP ($r = -0.433$, $p = 0.002$).

The decreased FRAP and GSH levels in plasma and bilirubin and albumin levels in serum of patients with active CD compared to inactive CD and controls underlines the importance of OxS in the pathophysiology and activity of CD.

Key words: Crohn's disease, oxidative stress, FRAP assay, glutathione, bilirubin, albumin.

Introduction

Crohn's disease (CD) is a chronic inflammatory disease of the entire gastrointestinal tract characterized by periods of exacerbations of symptoms and remissions. In the active phase patients suffer from abdominal pain, weight loss, chronic diarrhoea, and fever, and the course might be complicated with intestinal strictures, fistulas, intraabdominal abscess formation or malnutrition [1]. In the etiopathogenesis of CD interactions among various factors are discussed. The genetic predisposition, environmental conditions and disturbed intestinal microbiota play an important role in dysregulation of inflammatory response of the gastrointestinal tract [1].

The oxidative stress (OxS) imbalance with overproduction of reactive oxygen species (ROS) is implicated in the pathogenesis of CD [2–4]. In normal conditions, numerous antioxidants can delay or prevent oxidative damage caused by the presence of ROS. Antioxidants are classified into extracellular (present in plasma) and intracellular, which are enzymatic (e.g. superoxide dismutase, glutathione peroxidase, catalase), and non-enzymatic compounds (e.g. glutathione [GSH], bilirubin, albumin, uric acid, vitamins A, C, E) [5]. The excess of ROS production and reduced activity of protective antioxidants leads to the inflammation of intestinal mucosa with impaired its regenerative capacity and with subsequent increased vulnerability to damage [6]. The increased intestinal permeability enhances the toxic effects of environmental factors like many allergens and microorganisms [6].

The evaluation of the OxS severity in CD patients may have therapeutic implications. The specific antioxidant drugs or supplementation, could have the additional effects especially in patients who do not fully adhere to the standard CD therapy [4]. In patients with active CD ROS are generated by resident phagocytic cells, microvascular endothelial cells, and mucosal epithelial cells in the inflammated intestine [7]. ROS damages polyunsaturated fatty acids, inducing lipid peroxidation and oxidative damage which have been implicated as important mechanisms for cell injury and death in CD [6, 8, 9].

Antioxidant defences, such as the ferric reducing ability of plasma (FRAP), GSH, bilirubin, albumin and other are crucial in limiting cell injury induced by

ROS [5, 10, 11]. The persistence of OxS affects the course of CD and is associated with characteristic features of the disease, like transmural inflammation and complications [2, 6–8, 10].

The aim of this study was to examine whether nonenzymatic antioxidants are associated with active CD, by using the FRAP and GSH assay in plasma. Additionally, we measured bilirubin and albumin levels as two individual components of the plasma antioxidant system.

Materials and Methods

The study included 55 adult patients (21 women and 34 men) with confirmed CD and recruited from the gastroenterology clinic of the University Hospital in Krakow. The CD patients were divided into two subroups based on the CD activity index (CDAI): group I included 30 patients with active CD (CDAI ≤150 (age range, 19–62 years; mean age, 31.4 ± 6.1 years), and group II included 25 patients with nonactive CD (CDAI ≥150; mean age, 28.9 ± 8.1years) [12, 13].

The III group consisted of 25 age- and sex-matched patients with functional disorders of the gastrointestinal tract (control group; age range 19–66 years; mean age, 32.6 ± 7.2 years).

The exclusion criteria were as follows: diabetes, chronic inflammatory disease, active infection, history of cancer, chronic hepatic and hepatobiliary diseases, chronic renal failure, alcohol abuse, smoking, the use of antibiotics, antioxidants, or anti-inflammatory medications in the past 6 months, pregnancy or lactation.

CD diagnosis was made based on the clinical, endoscopic, radiological and histopathological criteria [1]. The activity of CD was determined using the CD Activity Index (CDAI) [1, 12, 13]. CDAI is a complex scoring system covering clinical symptoms (severity of abdominal pain, number and consistency of stools, patient general activity), laboratory tests (e.g. albumin, hematocrit values), perianal changes and extra intestinal manifestation of CD. CDAI values <150 indicate CD remission, and values of >150 indicate active CD (150–219, mild exacerbation; 220–450, moderate; >450, severe exacerbation) [1, 12]. Patients were treated with azathioprine (2–2.5 mg/kg/day) [14].

The following variables were evaluated: clinical features, CD history and location, results of gastrointestinal tract examinations, measurements of weight and height, and CDAI.

The study was conducted in accordance with the Declaration of Helsinki. The protocol was approved by the Bioethics Committee of Jagiellonian University in Krakow, Poland (KBET/200/B/2014). All individuals provided written informed consent to participate in the study.

Blood tests

Fasting blood samples were collected from the antecubital vein. On the same day, the laboratory parameters were determined: complete blood count, albumin, bilirubin, and CRP levels in serum in the hospital laboratory. Complete blood count was performed with a Sysmex XE-2100 hematology automated analyzer (Sysmex, Kobe, Germany). CRP, bilirubin and albumin were assayed using a Modular P clinical chemistry analyzer (Roche Diagnostics, Mannheim, Germany). Blood samples were centrifuged at 3000 × *g* for 10 min at 4°C, and plasma samples were stored at –80°C for determination of FRAP and GSH.

FRAP assay

FRAP level in plasma was measured according to the method of Benzie and Strain, as described previously [15, 16]. The plasma samples were mixed with 3 ml reagent mixture containing acetate buffer (pH 3.6), 5 mM tripyridyltriazine in 40 mM HCl, and 20 mM ferric chloride. FRAP values were calculated by preparing an aqueous solution of known FeII ($FeSO_4 \cdot 7H_2O$) concentration in the range of 0–1.0 mM and the blank contained FRAP reagent mixture. Absorbance was assessed at a wavelength of 593 nm (FLUOstar Omega spectrophotometer; BMG Labtech). FRAP values were expressed as mmol/mg of protein.

GSH assay

GSH level in plasma was measured using the Ellman method [17], as described previously [16, 18]. Briefly, the reaction of thiols with the chromogen dithiobis-nitrobenzoic acid formatted the yellow dianion of 5-thio-2-nitrobenzoic acid, the supernatant was introduced and after 5 min the absorbance was read at room temperature at a wavelength of 412 nm (FLUOstar Omega spectrophotometer; BMG Labtech). GSH concentrations were expressed as nmol/mg of protein.

Total protein assessment

Concentrations of total protein were measured using the bicinchonic acid (BCA) method according to the manufacturer's instructions (Sigma-Aldrich, USA), as described previously [16, 19]. The BCA method entails reducing Cu^{2+} to Cu^{+}, and Cu^{+} ions react in an alkaline medium with BCA, which gives it a violet colour. Absorbance was read at a wavelength of 562 nm at 37°C (FLUOstar Omega spectrophotometer; BMG Labtech), and a calibration curve was plotted and a simple linear regression equation derived. Protein concentrations were expressed in mg/mL.

Statistical analysis

Statistical analyses were conducted using the Statistica 10.0 software (StatSoft, Tulsa, OK, USA). Data were presented as number of patients and percentages for categorical variables, as means with standard deviations for normally distributed continuous variables, and as medians with interquartile ranges for nonnormally distributed continuous variables. The Shapiro-Wilk test was used to assess the normality of the data distribution, and for the comparison of normally distributed variables between the groups, the Student's *t*-test was used. The statistical significance between the groups was determined by the Kruskal-Wallis analysis of variance test, and the Mann-Whitney *U* test was then used where applicable. Associations between multiple variables were assessed using the Spearman rank correlation coefficient. A significance level was defined as a *P* value of less than 0.05.

Results

The characteristics of the groups are presented in Table 1. Patients with active CD were characterized by a lower hemoglobin concentration compared with those with inactive CD and controls ($p < 0.01$). The platelet count and serum CRP level were higher in patients with active CD than those with inactive CD and controls. The mean CDAI score of patients with active CD was 268.5 ± 26.1 versus 87.2 ± 9.4 for those with inactive disease ($p < 0.01$). Twenty nine patients had mild CD activity (CDAI 150–219 points), and 15 had moderate CD activity (CDAI 220–450 points). In the majority of patients with CD (68%), disease-associated lesions were located both in the small intestine and in the colon. Enterocutaneous and enteroenteric fistulas and abscesses were present in 52% of patients with exacerbated CD, while subjects in remission showed no active fistulas or abscesses.

Table 1. Demographic characteristics of the controls and the study subgroups of patients with active and inactive Crohn's disease.

	Controls (n = 25)	Patients with CD (n = 55)	
		Active CD (n = 30)	Inactive CD (n = 25)
	Mean ± SD	Mean ± SD	Mean ± SD
Age, years	32.6 ± 7.2	31.4 ± 6.1	28.9 ± 8.1
Women, n (%)	14 (56%)	18 (60%)	12 (48%)
Disease duration, years		7.1 ± 2.6	9.2 ± 3.8
BMI, kg/m^2	23.1 ± 2.5	22.8 ± 1.9	23.6 ± 2.1
CDAI, points		268.5 ± 26.1[a]	87.2 ± 9.4

Abbreviations: CD = Crohn's disease, BMI = body mass index, CDAI = Crohn's disease activity index.
[a] $p = 0.01$ compared with inactive CD.

Bilirubin, albumin, FRAP and GSG concentrations

The bilirubin levels were significantly lower ($p < 0.01$) in patients with active CD than in inactive CD ($p = 0.01$) and controls ($p = 0.05$) (8.3 ± 0.9, 12.5 ± 1.8, 11.7 ± 2 µmol/l, respectively). Albumin concentration in serum was significantly lower in patients with active CD in comparison to inactive CD (33.6 ± 3.2 g/l and 43.2 ± 3.5 g/l; $p < 0.005$) and controls (44.0 ± 3.9 g/l, $p < 0.05$) (Table 2).

Table 2. Results of laboratory test in the controls and the study subgroups of patients with active and inactive Crohn's disease.

	Controls (n = 25)	Patients with CD (n = 55)	
		Active CD (n = 30)	Inactive CD (n = 25)
	Mean ± SD	Mean ± SD	Mean ± SD
Hemoglobin, g/dl	13.5 ± 1.1	11.3 ± 0.9[ab]	13.1 ± 1.1
Erythrocytes, $\times 10^6$/µl	4.2 ± 0.3	3.9 ± 0.2	4.1 ± 0.4
Leukocytes, $\times 10^3$/µl	6.0 ± 0.8	7.2 ± 1.2	5.9 ± 1.4
Platelets, meadian (IQR), $\times 10^3$/µl	237.1 (215.2–300.4)	389.7 (241.2–458.6)[bc]	245.3 (225.1–296.5)
CRP, mg/dl, meadian (IQR), mg/l	1.7 (0.8–4.1)	37.1 (17.9–49.8)[d]	2.3 (0.7–4.2)
Bilirubin (µmol/l)	12.5 ± 1.8	8.3 ± 0.9[ab]	11.7 ± 2.0
Albumin (g/l)	44.0 ± 3.9	33.6 ± 3.2[bd]	43.2 ± 3.5

Data are presented as mean ± standard deviation (SD) unless otherwise stated.
Abbreviations: CD = Crohn's disease, CRP = C-reactive protein.
[a] $p = 0.01$ compared with inactive CD; [b] $p = 0.05$ compared with control; [c] $p = 0.05$ compared with inactive CD; [d] $p = 0.005$ compared with inactive CD and control.

FRAP levels in the plasma of CD patients and controls are presented in Table 3. There were significant differences in FRAP levels between patients with active and inactive CD (0.01 (0.02); and 0.018 (0.02), respectively) and control group (0.02 (0.02); $p < 0.05$). GSH levels in plasma of patients with active CD were significantly lower than those with inactive CD and controls (5.4 (8.0), 7.4 (5.2) and 11.5 (7.3), respectively; $p < 0.05$).

Spearman's rank correlations analyses were performed between FRAP, GSH, bilirubin and albumin concentrations as OxS parameters in plasma and BMI, CDAI, CRP, hemoglobin, platelet count, and white cell count in patients with active CD.

As shown in Table 4, positive correlations were detected between FRAP levels and hemoglobin level. Negative correlations were found between FRAP levels in plasma and CDAI, CRP and platelet count. Positive correlations were observed between the GSH and hemoglobin levels. Negative correlations were detected between GSH concentrations in plasma and CDAI value, CRP levels and platelet count.

Table 3. Plasma levels of ferric reducing ability of plasma and glutathione in controls and patients with active and inactive Crohn's disease.

	Controls (n = 25)		Patients with CD (n = 55)			
			Active CD (n = 30)		Inactive CD (n = 25)	
	Median (IQR)	Min-max	Median (IQR)	Min-max	Median (IQR)	Min-max
FRAP (mmol/g protein)	0.02 (0.02)	0.02–0.03	0.01[a] (0.02)	0.01–0.03	0.018 (0.02)	0.01–0.02
GSH (μmol/g protein)	11.5 (7.30)	6.40–13.9	5.40[a] (8.00)	3.70–10.1	7.400 (5.20)	3.20–10.0

Abbreviations: CD — Crohn's disease, IQR — 75th percentile, FRAP — ferric reducing ability of plasma, GSH — glutathione, p — Kruskal-Wallis test with post-hoc analysis; [a] $p < 0.05$ compared with inactive CD and controls.

Table 4. Correlations between antioxidants (FRAP, GSH, bilirubin and albumin) and clinical and laboratory parameters of patients with active Crohn's disease.

		BMI	CDAI	CRP	Hb	PLT	WBC
FRAP (n = 30)	r	0.308	−0.572	−0.457	0.351	−0.411	−0.220
	p	0.073	<0.003	0.017	0.047	0.003	0.151
GSH (n = 30)	r	0.294	−0.761	−0.409	0.470	−0.519	−0.286
	p	0.061	0.001	0.007	0.001	0.004	0.093
Bilirubin (n = 30)	r	−0.290	−0.328	−0.395	0.302	0.281	0.253
	p	0.081	0.036	0.013	0.049	0.075	0.129
Albumin (n = 30)	r	0.215	−0.518	−0.483	0.372	0.297	0.163
	p	0.353	0.002	0.002	0.020	0.261	0.307

Abbreviations: BMI — body mass index. CDAI — Crohn's disease activity index, CRP — C-reactive protein, Hb — hemoglobin, PLT — platelet count, WBC — white blood cell count.
Data represents: r — Spearman's rank correlation coefficient, p — significance level, n — number of observations.

There were negative correlations between bilirubin levels and CDAI value and CRP levels, and positive correlations with hemoglobin concentrations in the blood (Table 3). Serum albumin levels in patiens with active CD negatively correlated with CDAI and CRP and positively with hemoglobin concentrations.

Discussion

In the present study, the antioxidative parameters in plasma of patients with active CD were significantly lower compared to those with inactive CD and controls. The antioxidant levels in the plasma of both FRAP and GSH, as well as bilirubin and albumin concentrations in serum were significantly decreased in active CD patients compared to inactive CD patients and controls. Low FRAP, GSH, bilirubin and albumin levels were negatively correlated with CD activity assessed by CDAI as well as with selected biochemical markers of inflammation.

OxS is involved in the pathogenesis of numerous diseases including CD and constitutes an important pathogenetic mechanism in CD and its complications [6, 8]. Estimating total antioxidant capacity as a clinical marker of OxS is based on determining the concentration of antioxidants in bodily fluids [3, 20, 21]. Activities of low molecular weight antioxidants (e.g., alpha-tocopherol, ascorbic acid, glutathione, uric acid, bilirubin), proteins (e.g., ceruloplasmin, albumin, transferrin), and enzymatic systems contribute to the total plasma antioxidant capacity [2, 22–24]. The FRAP level is correlated with the risk of certain diseases and their complications, including CD [22, 25].

The biological roles of GSH, as a major intracellular antioxidant enzyme, include protecting against OxS, controlling the reduction of SH groups in intracellular proteins, regulating apoptosis, maintaining other antioxidants in their reduced forms, participating in the synthesis of DNA, proteins, leukotrienes, and prostaglandins, and forming endogenous conjugates [3, 20]. A disturbance in GSH homeostasis contributes to the pathogenesis of several diseases. Therefore easuring the GSH level is important for exploring the pathogenesis of inflammatory diseases. CD is associated with decreased levels of GSH in intestinal mucosa, which supports a role for OxS in this disease [2, 8].

The effects of OxS on CD are intensified by lipid peroxidation, and the role of malonyldialdehyde (MDA) as a marker of lipid peroxidation was recently widely studied [2, 16, 25–27]. Jahanshahi *et al.* showed that levels of thiobarbituric reactive substances (TBARS), markers of lipid peroxidation, were significantly increased in the saliva of CD patients compared to those with ulcerative colitis [28]. This was confirmed by Alzoghaibi *et al.* who showed higher plasma levels of MDA in CD patients than in controls [29]. Our former study have confirmed that MDA assessed in saliva or serum may be a valuable marker in evaluation of the clinical severity of CD [16]. A greater understanding of the role of OxS in CD may facilitate the development of antioxidant therapies that may protect intestinal enterocytes against destruction [5, 7, 30].

Several authors have found the reduced serum bilirubin and albumin concentrations in CD patients, which are the endogenous components of the blood antioxidant

system [5, 31]. Bilirubin protects against lipid peroxidation and Schieffer *et al.* suggested that low serum bilirubin is mediated by the inflammation and this phenomenon was observed not only in CD but also in various inflammatory diseases [32, 33]. The other important circulating antioxidant is albumin [5], which is a marker of nutritive status and is widely involved in many bioactive functions [34, 35]. Albumin represents a very abundant and important circulating antioxidant. These properties are attributed to its unique biochemical structure of albumin with ligand binding and free radical-trapping activities [35, 36].

In this study, antioxidant activity of the blood, measured using FRAP, GSH, bilirubin and albumin levels, was significantly reduced in CD. FRAP levels in plasma were significantly lower in active and inactive CD patients compared to controls. GSH levels in plasma were also significantly decreased in patients with active CD compared to inactive CD and controls. Thus, patients with active CD had significantly diminished antioxidant activity.

Bilirubin is the potent antioxidant of lipid peroxidation products [2, 3, 32]. The recent clinical study indicated that CD patients may have increased bilirubin metabolism that may result in reduced circulating total serum bilirubin [33]. An Australian study reported that bilirubin levels were significantly lower in severe asthma, suggesting altered regulation of inflammation in asthmatics by antioxidant vitamins and bilirubin [37]. This observation is consistent with our results, indicating the relationship between the altered serum concentration of bilirubin and oxidative imbalance. However, the role of bilirubin in OxS in CD requires further research.

In our study, antioxidant activity was decreased in patients with active CD and increased in those in remission. This is consistent with previous reports that patients with active CD have elevated OxS and reduced antioxidant activity in serum, whereas these parameters in patients in remission are comparable to those of healthy controls [10, 22, 24].

The plasma FRAP and GSH levels of CD patients show considerable variation [25]. Therefore, it is important to consider all factors that affect OxS markers, including duration of CD, clinical status, medications, sample type (e.g. serum, plasma, other body fluids), time of sample collection. Moreover, our results suggest that FRAP and GSH in plasma and bilirubin and albumin in serum have diagnostic value and could be used as prognostic markers of OxS in CD patients. Further study with a larger population would increase the statistical power of the tests and the value of the research.

This study has several limitations. First, evaluating a larger number of patients would increase the value of the study. Second, other OxS and antioxidant parameters were not studied. Third, all patients with CD were treated chronically with azathioprine, and we cannot rule out an effect of this medication on the results.

In conclusion, the decreased antioxidant activity in plasma confirm that the OxS level is correlated with the severity of CD. The decreased FRAP and GSH in plasma and both bilirubin and albumin levels in serum of patients with active CD return to normal ranges on clinical remission. These findings also suggest that not only serum but also a plasma has also the potential to assess antioxidant activity in CD patients, particularly using FRAP and GSH as markers.

Acknowledgements

This study was supported by grant from the specific subsidy for holding the research capacity of the Ministry of Science and Higher Education (grant no. K/ZDS/007169). The funding body had no role in this study or its publication.

Contribution statement

KS and WK conceived the idea of and designed the research, KS and TM carried out the literature research and study selection, the qualities of included studies were carried out by KK, HP, DO, JP-P, DO. KS and WK analyzed the data, KS and TM wrote the paper and revised the manuscript for final submission

Conflict of interest

None declared.

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