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Antioxidant Status of the Celiac Mucosa: Implications for Disease Pathogenesis

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1. Introduction

Aerobic organisms require ground state oxygen to live. However, the use of oxygen during normal metabolism produces reactive oxygen species (ROS), some of which are highly toxic and deleterious to cells and tissues because of the ability to react with and alter all principal molecules of the cell, including lipids, proteins, carbohydrates and nucleic acids. It has been estimated that a human cell is affected by 1.5×10^5 oxidative strokes per day (Beckman & Ames, 1997). Under normal conditions, damage by oxygen radicals is kept in check by an efficient array of antioxidant (AO) mechanisms, such as AO enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR), as well as nonenzymatic scavengers. Although potentially deleterious, ROS also have important beneficial functions as a part of protective mechanism against microorganisms and serving as cell signaling molecules.

1.1 Reactive oxygen species (ROS)

The term "reactive oxygen species" is used for a group of chemical species that contain oxygen and are characterized by a high reactivity towards inorganic molecules, as well as biomolecules. ROS are:

- molecules, such as hydrogen peroxide (H_2O_2),
- ions, such as hypochlorite anion (OCl^-),
- free radicals, like hydroxyl radical ($OH\cdot$),
- superoxide anion radical, which is an anion and a radical ($O_2\cdot^-$).

Free radicals are molecules that contain one or more unpaired electrons in outer orbit (Halliwell & Gutteridge, 1989). The presence of the unpaired electron makes them highly reactive, which means that they can react with the majority of surrounding molecules

including proteins, lipids, carbohydrates and nucleic acids. Tending to obtain a stable state, they "attack" the nearest stable molecule "stealing" an electron. When the "attacked" molecule loses an electron, it becomes a free radical itself, beginning a chain reaction that may end with the destruction of a living cell. The most important free radicals in biological systems are O_2^- , $OH\cdot$, nitric oxide ($NO\cdot$), lipid peroxy radical ($ROO\cdot$) and alkoxy radical ($RO\cdot$).

1.1.1 The activation of oxygen

Atmospheric oxygen in its basic state is unique among other gaseous elements, because it is a biradical, which means that in its outer orbit it has two unpaired electrons with parallel spins (a "triplet state" 3O_2). Due to this feature oxygen can hardly react with organic molecules, unless it is previously activated (Eltner, 1987). If a biradical form of oxygen absorbs energy sufficient to change the spin of one of the unpaired electrons, a "singlet state" ($^1O_2^*$) is generated, having two electrons with opposite spins. The oxygen thus activated can participate in reactions involving simultaneous transfer of two electrons (divalent reduction). Since paired electrons are usual in organic molecules, singlet oxygen is more reactive towards organic molecules than oxygen in the basic state. The other mechanism of activation is a gradual monovalent reduction of oxygen, generating O_2^- , H_2O_2 , $OH\cdot$ and in the end H_2O .

1.1.2 Sources of ROS

Free radicals and ROS can originate via action of various endogenous and exogenous factors. Endogenous sources of ROS are autoxidation of different organic and inorganic molecules, enzymatically catalyzed oxidation and the "respiratory burst". Superoxide anion radical is the most common oxidant produced by normal cell metabolism. The main sources of O_2^- are electron transport systems in membranes of mitochondria and other organelles (endoplasmic reticulum, chloroplasts). Apart from the mitochondrial respiratory chain, an array of nonenzymatic and enzymatic reactions can be the source of ROS. Autoxidation of various cell molecules (quinones, thiols, flavines, catecholamines, hemoglobin, myoglobin) produce O_2^- . Ferrous ions are also subjected to autoxidation followed by ROS production. ROS may be a direct product of enzymatic reactions. Myeloperoxidase in neutrophils in the presence of chloride produces OCl^- from H_2O_2 . Xanthine oxidase (XO) catalyzes oxidation of hypoxanthine to xanthine and xanthine to uric acid, producing O_2^- and H_2O_2 (Valko et al., 2004). Certain cells of the immune system (neutrophils, eosinophils, mononuclear phagocytes, B lymphocytes) during phagocytosis produce ROS (OCl^- , $OH\cdot$, $^1O_2^*$ or chloramines) as microbicidal agents. A precursor of more reactive oxidants is O_2^- , whose production is associated with increased oxygen consumption in these cells sometimes even up to 50 times and this metabolic process is known as the "respiratory burst" (Babior, 1984).

Exogenous sources of ROS are drugs, radiation and smoking. Certain drugs such as some antibiotics and antineoplastic agents (anthracyclines, methotrexate) may increase ROS production under hyperoxic conditions (Gressier et al., 1994). In addition, components of some drugs may deplete AO reserves, enhancing effects of lipid peroxidation (Grisham et al., 1992). Radiotherapy can cause free radical production. Electromagnetic and particle irradiation produce primary radicals transferring their energy to the cell molecules. Tobacco smoke contains a great amount of oxidants. It has been suggested that 10^{14} different oxidants

such as aldehydes, epoxides, peroxides, quinones, hydroquinones, NO \cdot etc., are being imported in organism by just one breath of cigarette smoke (Church & Pryor, 1985).

1.1.3 Effects of ROS

One of the most important effects of ROS is oxidative damage of polyunsaturated fatty acids (PUFA) in cell membrane lipids. This process is known as lipid peroxidation (LPO). It is a chain reaction that provokes changes in the membrane phospholipid bilayer structure and modifies membrane proteins, causing loss of membrane elasticity and selective permeability and disruption of its other functions (Spiteller, 2007). The damage provoked by LPO can be prevented by chain-breaking antioxidants (β -carotene, lycopene, vitamins A, C, E). Strong oxidants, such as OH \cdot can react with all components of the DNA molecule, causing single- or double-strand breaks and an increased rate of mutations (Egler et al., 2005). Permanent modifications of the genetic material represent the first step towards mutagenesis, carcinogenesis and aging. Proteins can also be oxidized by strong oxidants. Amino acids containing sulfur, especially the thiol group (-SH), are particularly susceptible to ROS. Oxidants can activate or inactivate proteins by oxidizing -SH groups and modifying amino acids (Davies, 1987). As a consequence of the deleterious effects of ROS, necrotic cell death may ensue. In addition, ROS and changes in cellular redox state may play a crucial role in the regulation and initiation of processes associated with apoptosis (Kroemer et al., 1998; Mignotte & Vayssiere, 1998).

Although a great importance is given to the negative effects of ROS, they also have beneficial physiological functions in the cell. Their role in the defense against microorganisms is indispensable. During phagocytosis activated inflammatory cells produce ROS to kill microbes. ROS can also have a critical role in signal transduction and redox regulation of gene expression (Thannickal & Fanburg, 2000) and the regulation of cell growth and proliferation (Burdon, 1996).

1.2 Antioxidant defense system

Detoxification of ROS is a sine qua non of aerobic life, hence a complex AO system has evolved due to evolutionary pressures. Antioxidants are agents which scavenge ROS, inhibit their production and/or repair the damage they have caused (Halliwell, 1991). The AO system involve AO enzymes (SOD, CAT, GPx), nonenzymatic antioxidants (glutathione (GSH), vitamin C, vitamin E, β -carotene, flavonoids), auxiliary enzymes that regenerate active forms of antioxidants (GR, glutathione-S-transferase (GST), glucose-6-phosphate dehydrogenase (G6PDH)), as well as metal binding proteins (transferrin, ceruloplasmin, albumin).

SOD catalyzes dismutation of O $_2^{\cdot-}$ to H $_2$ O $_2$ and oxygen. This reaction is 10 4 times faster than spontaneous dismutation. In humans three different forms of this enzyme exist: cytosolic or copper, zinc SOD (CuZnSOD), mitochondrial or manganese SOD (MnSOD) and extracellular SOD (ECSOD). Catalase catalyzes decomposition of H $_2$ O $_2$ to water and oxygen.

The glutathione redox cycle is a key mechanism for protection of cell membranes from damage by LPO. This cycle involves enzymes GPx, which uses GSH to reduce organic peroxides and hydrogen peroxide and GR, which reduces the oxidized form of glutathione (GSSG) with concomitant oxidation of NADPH. In a wider sense this cycle also includes the

enzymes that synthesize GSH (γ -glutamylcysteine synthetase and GSH synthetase), G6PDH, which regenerates NADPH, as well as GST. GSH is a potent antioxidant, antitoxin and enzymatic cofactor. It can directly react with free radicals. GSH has also an important role in regenerating active forms of other antioxidants such as vitamins E and C and carotenoids (Jones et al., 2000).

1.3 The role of oxidative stress in the pathogenesis of gastrointestinal diseases

The oxidant versus AO balance may be altered in various pathological conditions, primarily or secondarily. If ROS production overwhelms the AO defense capacity of a cell, oxidative damage occurs and the condition is known as oxidative stress. Oxidative stress plays an important role in the pathogenesis of many diseases including various gastrointestinal disorders. It has been shown that the concentration of ROS is elevated in patients with various liver diseases such as alcoholic hepatitis and cirrhosis, while antioxidant therapy has protective effects in animal models of these disorders (Dryden et al., 2005). Colon cancer, as well as acute and chronic pancreatitis, have also been associated with oxidative stress (Dryden et al., 2005; Opara, 2003). It has been suggested that oxidative stress may have an important role in the pathogenesis of acquired megacolon, since decreased AO levels provoke changes in the intestinal levels of inhibitory neurotransmitters in patients affected by the disease (Koch et al., 1996). Necrotizing enterocolitis, a severe disorder found in infants, is another disease whose pathogenesis is attributed to oxidative stress (Otamiri & Sjö Dahl, 1991). *Helicobacter pylori* infection, an important factor in the pathogenesis of gastric cancer, is also followed by an increased production of ROS (Farinati et al., 2003).

The role of oxidative stress in pathological changes in gastrointestinal tract has mostly been studied in inflammatory bowel disease (IBD) such as ulcerative colitis and Crohn's disease. It has been suggested that oxidative stress plays an important role in the initiation, as well as progression of IBD and antioxidant therapy, for e.g. use of green tea polyphenols or SOD, significantly attenuates the disorder (Dryden et al., 2005). IBD is characterized by elevation in mucosal inflammatory cells, leading to disruption of the epithelial barrier. This allows highly immunogenic bacterial antigens, present in the intestinal lumen in high concentrations, to enter the normally sterile subepithelial layers, activating a cascade of destructive immunologic responses (Rezaie et al., 2007). Various theories regarding the initiation of the inflammatory response in the intestinal mucosa have been proposed, but none of them is universally accepted (Hendrickson et al., 2002). Many studies have reported the increased concentrations of oxidized biomolecules and the decreased concentrations of various antioxidants in patients affected by IBD, not only in the intestinal mucosa, but also in other parts of the gastrointestinal tract, as well as in the blood and respiratory system (Rezaie et al. 2006). It is known that oxidizing agents can induce clinical and histological alterations characteristic of IBD (Bilotta & Wayne, 1989; Meyer et al. 1981). ROS may damage intestinal mucosa and increase its permeability (Rao et al., 1997; Riedle & Kerjaschki, 1997). In addition, it has been shown that patients affected by Crohn's disease in latent phase, as well as their first-degree relatives, have increased intestinal permeability without inflammation. The fact that oxidative stress is present in the bowel before the beginning of the inflammatory cascade suggests that ROS are not collateral products of the inflammatory process, but play an important role in the pathogenesis of the disease (Buhner et al. 2006; Fries et al., 2005).

1.4 Oxidative stress and antioxidant status of patients with celiac disease

There is a growing body of evidence indicating that oxidative stress and the cellular redox status are also implicated in the pathogenesis of celiac disease. The results of various investigations suggest that gliadin disturbs the pro-oxidant-antioxidant balance in small intestinal mucosa of affected persons through overproduction of ROS (Boda et al., 1999; Dugas et al., 2003). Several *in vitro* studies have also reported redox imbalance and increased levels of free radicals after exposure of cells to gliadin (Dolfini et al., 2002; Maiuri et al., 2003; Rivabene, 1999; Tucková et al., 2002).

Data concerning the AO status of celiac patients are scarce. The results of a few investigations indicate that the AO capacity of celiac patients is diminished. Odetti et al. (1998) found a lowered level of vitamin E and increased levels of markers of oxidized lipids and proteins in the plasma of celiac patients subjected to gluten free diet, while Ståhlberg et al. have reported decreased GSH concentrations and GPx activity in erythrocytes and the small intestinal mucosa of children affected by celiac disease (Ståhlberg et al., 1988; Ståhlberg & Hietanen, 1991). In our previous papers (Stojiljković et al., 2007; Stojiljković et al., 2009) we showed that oxidative stress is strongly associated with CD and that the AO capacity of celiac patients is weakened by a depletion of GSH and reduced activities of GSH-dependent AO enzymes GPx and GR. In this study we describe the results of our investigation regarding the AO status of celiac patients with different degrees of severity of the mucosal lesion. The activities of AO enzymes MnSOD, CuZnSOD, CAT, GPx and GR, as well as the concentrations of GSH and lipid hydroperoxides (LOOH) were examined.

2. Materials and methods

2.1 Subjects

The study involved small intestinal biopsies from 55 children affected by celiac disease (24 boys, 31 girls; median age 8 years; range 1.5-16 years) who were attended at the University Children's Hospital, Belgrade, Serbia, between September 2003 and December 2006. Clinical characteristics of the patients are described in Table 1. Twenty six children were diagnosed in early childhood and by the time of sampling, they had been subjected to gluten-free diet (GFD) for 2-4 years. In the other 29 children, who were using gluten containing diet, the diagnosis was made at the time of the study. Among them, 18 children had active form of the disease with typical symptoms (chronic diarrhea, fatigue, failure to thrive or weight loss), while 11 children were asymptomatic. Typical villous atrophy was found on examination of intestinal biopsy specimens in all children on the gluten containing diet. The diagnosis of celiac disease was based on the revised criteria of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (Walker-Smith, 1990). The Ethical Committee of the Faculty of Medicine, University of Belgrade, approved the study. The parents of all patients included in the study gave written informed consent.

Histological evaluation was performed according to the modified Oberhuber-Marsh classification (Oberhuber et al., 1999). Patients were divided in 4 groups. In the Marsh 0 group, the mucosa was normal with no signs of inflammation (n = 17, all on GFD). In the Marsh 1+2 group, the mucosa was characterized by intraepithelial lymphocytosis (Marsh 1) or intraepithelial lymphocytosis accompanied by crypt hyperplasia (Marsh 2) (n = 9, six Marsh 1 and three Marsh 2, all on GFD). In the Marsh 3a group (n=20, seven asymptomatic

and 13 with active celiac disease) partial villous atrophy was present, while in the Marsh 3b group (n=9, four asymptomatic and five with active form of the disease) subtotal villous atrophy was found.

	Patients		
	active	asymptomatic	GFD
Number	18	11	26
Gender			
Male	6	6	12
Female	12	5	14
Age (years)			
Range	1,5-15	1,5-16	5-16
Median	4,63±1,19	8,34±1,36	8,78±1,24
Body mass index			
<15th percentile	10	3	5
15-75th percentile	7	8	13
>75th percentile	1	-	8
Hemoglobin (g/L) ¹	102.8±3.6	119.2±4.0 **	133.3±4.1*** #
Mean cell volume (fL) ²	70.1±2.6	73.8±3.5	76.7±1.6*
serum iron (µmol/L) ³	5.5±0.7	10.9±2.6**	17.6±3.5*** #

¹Normal value: 120-170 g/L

²Normal value: 86-98 fL

³Normal value: 10-22 µmol/L

Table 1. Clinical characteristics of patients affected by celiac disease. Hemoglobin, mean cell volume and serum iron are means ± SEM. Statistical significance: *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, significantly different from active patients; # $P < 0.05$ significantly different from asymptomatic patients. GFD, patients on gluten-free diet.

2.2 Sample preparation

From each patient 6-8 proximal small intestinal biopsy specimens were obtained. Some of them were used for histopathological analysis and others were washed in ice-cold saline and frozen at -70 °C for SOD, CAT, GPx, GR, GSH and LOOH assays. One biopsy specimen from each patient was kept on -70 °C for the GSH assay, while others were thawed within a week and homogenized in 20 volumes of cold sucrose buffer pH 7.4. Homogenates were vortexed 3 times for 15 seconds and then kept at -70 °C. Thawed homogenates were centrifuged (Eppendorf centrifuge 5417R, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) at 8600 g, 4 °C, for 10 minutes. Supernatants were stored at -70 °C.

2.3 Assays

All assays were performed using the Perkin Elmer Lambda 25 Spectrophotometer (Perkin Elmer Instruments, Norwalk, CT, USA). The specific enzyme activities of SODs and CAT were expressed as units per milligram of protein (U/mg) and of GPx and GR as milliunits per milligram of protein (mU/mg). The GSH and LOOH concentrations were expressed in micromoles per liter (µmol/l).

SOD assay. Total SOD activity was measured using the Oxis Bioxytech® SOD-525™ Assay (Oxis International, Inc., Portland, OR, USA). The method is based on the SOD-mediated increase in the rate of autoxidation of reagent 1 (5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo[c]fluorene,

R1) in aqueous alkaline solution, yielding a chromophore with maximum absorbance at 525 nm. The kinetic measurement of the change in absorbance at 525 nm is performed. One SOD-525 activity unit is defined as the activity that doubles the autoxidation rate of the control blank. CuZnSOD activity was measured as described above, after pretreating samples with ethanol-chloroform reagent (5/3 vol/vol), which inactivates MnSOD. MnSOD activity was then calculated by subtracting CuZnSOD activity from total SOD activity.

CAT assay. CAT activity was measured by the method of Beutler (1982), which is based on the measurement of the rate of H₂O₂ decomposition by catalase from the examined samples. The decomposition of H₂O₂ was demonstrated by a decrease in absorbance at 230 nm as a function of time. One CAT activity unit is defined as 1 μ mol of H₂O₂ decomposed per minute under the assay conditions.

GPx assay. Gpx activity was determined by the Oxis Bioxytech® GPx-340™ Assay (Oxis International, Inc., Portland, OR, USA). Upon reduction of organic peroxide by GPx, oxidized glutathione (GSSG) is produced and its recycling to GSH by GR is accompanied by oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) to NADP⁺. The rate of NADPH oxidation, followed by a decrease in absorbance at 340 nm as a function of time, is directly proportional to the GPx activity in the sample. One GPx-340 activity unit is defined as 1 μ mol of NADPH consumed per minute under the assay conditions.

GR assay. The activity of GR was measured by the Oxis Bioxytech® GR-340™ Assay (Oxis International, Inc., Portland, OR, USA). The assay is based on the oxidation of NADPH to NADP⁺ by GR from the sample. The rate of NADPH oxidation was determined by the rate of a decrease in absorbance at 340 nm. One GR-340 unit is defined as 1 μ mol of NADPH oxidized per minute under the assay conditions.

GSH assay. The concentration of GSH was determined by the Oxis Bioxytech® GSH-420™ Assay (Oxis International, Inc., Portland, OR, USA). The thawed tissue was homogenized in 20 volumes of precipitating reagent (trichloroacetic acid) and homogenates were centrifuged at 3000 g, 4 °C, 10 minutes. Supernatants were used for GSH assay. The reaction is performed in three steps. The sample was first buffered and treated with the reducing agent (tris(2-carboxyethyl)phosphine) to reduce any oxidized glutathione present in the sample. Then the chromogen (4-chloro-1-methyl-7-trifluoromethylquinolinium methylsulphate) was added forming thioethers with all thiols from the sample. After addition of base to raise the pH over 13, a β -elimination specific to the GSH-thioether results in the chromophoric thione. The absorbance at 420 nm is directly proportional to the GSH concentration.

LOOH assay. The concentration of LOOH was determined by the Oxis Bioxytech® LPO-560™ Assay (Oxis International, Inc., Portland, OR, USA). The assay is based on the oxidation of ferrous to ferric ions by LOOH from the sample under acidic conditions. Ferric ions bind with the indicator dye (xylenol orange) and a stable colored complex is formed. The absorbance at 560 nm is directly proportional to the LOOH concentration. To eliminate H₂O₂ interference the samples were pretreated with catalase.

2.4 Statistics

Differences between the groups were tested by the Kruskal-Wallis test. Multiple comparisons of the groups were performed by the Dunn test. Correlations between AO

parameters and the degree of mucosal lesion were evaluated by the Spearman's rank order correlation coefficient r_s . A P value lower than 0.05 was considered significant.

3. Results

All parameters, except CuZnSOD and CAT activity, varied significantly between the analyzed groups: MnSOD: $H = 8.79$, $P < 0.05$; CuZnSOD: $H = 5.23$, $P > 0.05$; CAT: $H = 5.75$, $P > 0.05$; GPx: $H = 12.61$, $P < 0.01$; GR: $H = 9.81$, $P < 0.05$; GSH: $H = 32.70$, $P < 0.001$; LOOH: $H = 22.92$, $P < 0.001$ (Kruskal-Wallis test).

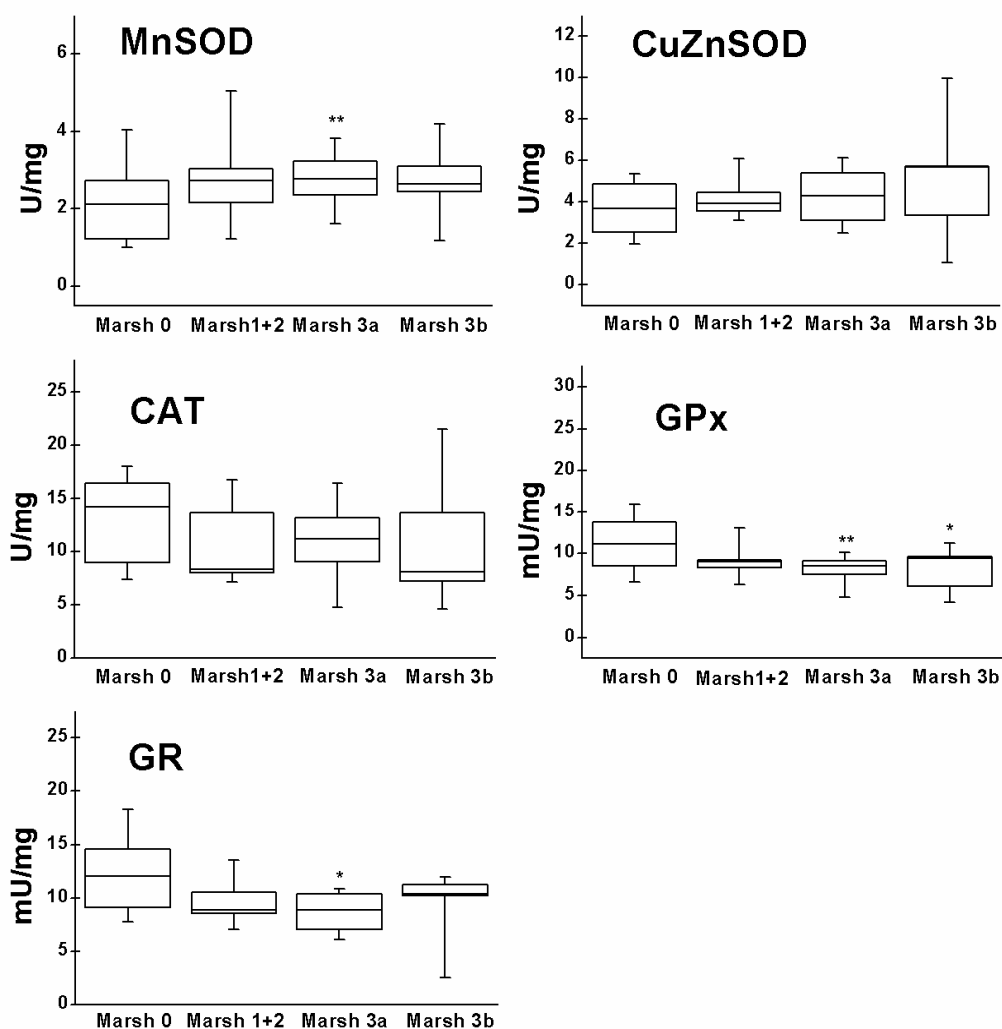


Fig. 1. The activities of manganese superoxide dismutase (MnSOD), copper-zink SOD (CuZnSOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) in normal intestinal mucosa (Marsh 0), mucosa with intraepithelial lymphocytosis or intraepithelial lymphocytosis accompanied by crypt hyperplasia (Marsh 1+2), mucosa with partial (Marsh 3a) or subtotal villous atrophy (Marsh 3b). Boxes represent values between 25th and 75th percentile. Medians are given inside the boxes. Whiskers extend between min and max values. ** $P < 0.01$, * $P < 0.05$, significantly different from Marsh 0 group.

The activities of the AO enzymes are represented in Figure 1. In comparison to Marsh 0 group, the MnSOD activity was significantly elevated in Marsh 3a group ($P < 0.01$). GPx activity was significantly lower in Marsh 3a ($P < 0.01$) and Marsh 3b groups ($P < 0.05$) than in Marsh 0. GR activity was also reduced in Marsh 3a ($P < 0.01$) when compared to Marsh 0.

The patients with villous atrophy (Marsh 3a and 3b) had significantly reduced GSH level in comparison to the patients with normal mucosa (Marsh 0, $P < 0.0001$) or milder mucosal lesion (Marsh 1+2, $P < 0.01$). Significant increase in LOOH levels was found in Marsh 1+2 ($P < 0.01$), Marsh 3a and 3b ($P < 0.001$), comparing to the patients with normal mucosa. LOOH concentration was also higher in patients with villous atrophy than in patients with Marsh 1+2 lesions ($P > 0.05$) (Figure 2).

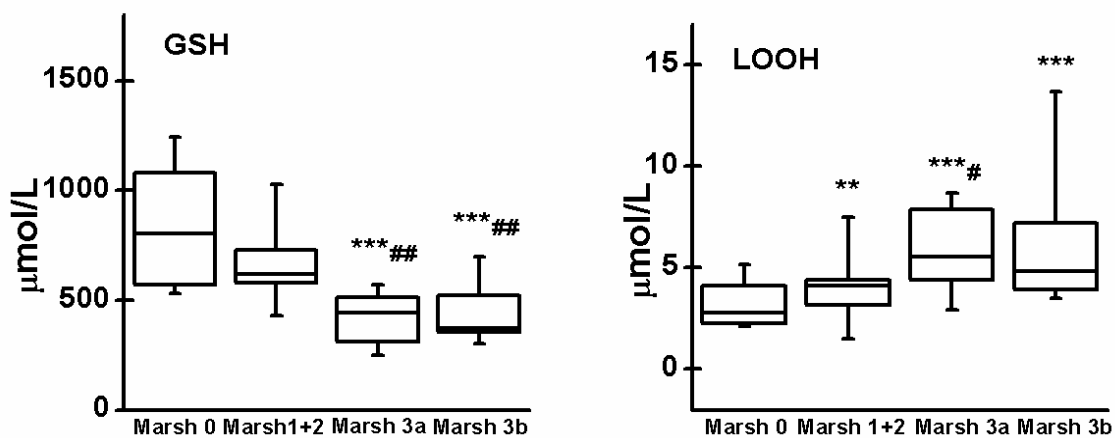


Fig. 2. Concentrations of glutathione (GSH) and lipid hydroperoxides (LOOH) in normal intestinal mucosa (Marsh 0), mucosa with intraepithelial lymphocytosis or intraepithelial lymphocytosis accompanied by crypt hyperplasia (Marsh 1+2), mucosa with partial (Marsh 3a) or subtotal villous atrophy (Marsh 3b). Boxes represent values between 25th and 75th percentile. Medians are given inside the boxes. Whiskers extend between min and max values. *** $P < 0.001$, ** $P < 0.01$, significantly different from Marsh 0 group; ## $P < 0.01$, # $P < 0.05$, significantly different from Marsh 1+2 group.

All investigated parameters correlated significantly with the degree of mucosal damage (Figure 3). Positive correlations were found between the severity of mucosal lesion and the activities of MnSOD ($r_s = 0.33$, $P < 0.01$), CuZnSOD ($r_s = 0.27$, $P < 0.01$) and CAT ($r_s = 0.25$, $P < 0.05$). On the contrary, GSH concentration as well as the activities of GSH-related enzymes GPx and GR inversely correlated with degree of the mucosal lesion ($r_s = -0.67$, $P < 0.0001$; $r_s = -0.40$, $P < 0.001$; $r_s = -0.27$, $P < 0.05$, respectively). In addition, significant positive correlation was found between the LOOH level and the degree of mucosal damage ($r_s = 0.56$, $P < 0.0001$).

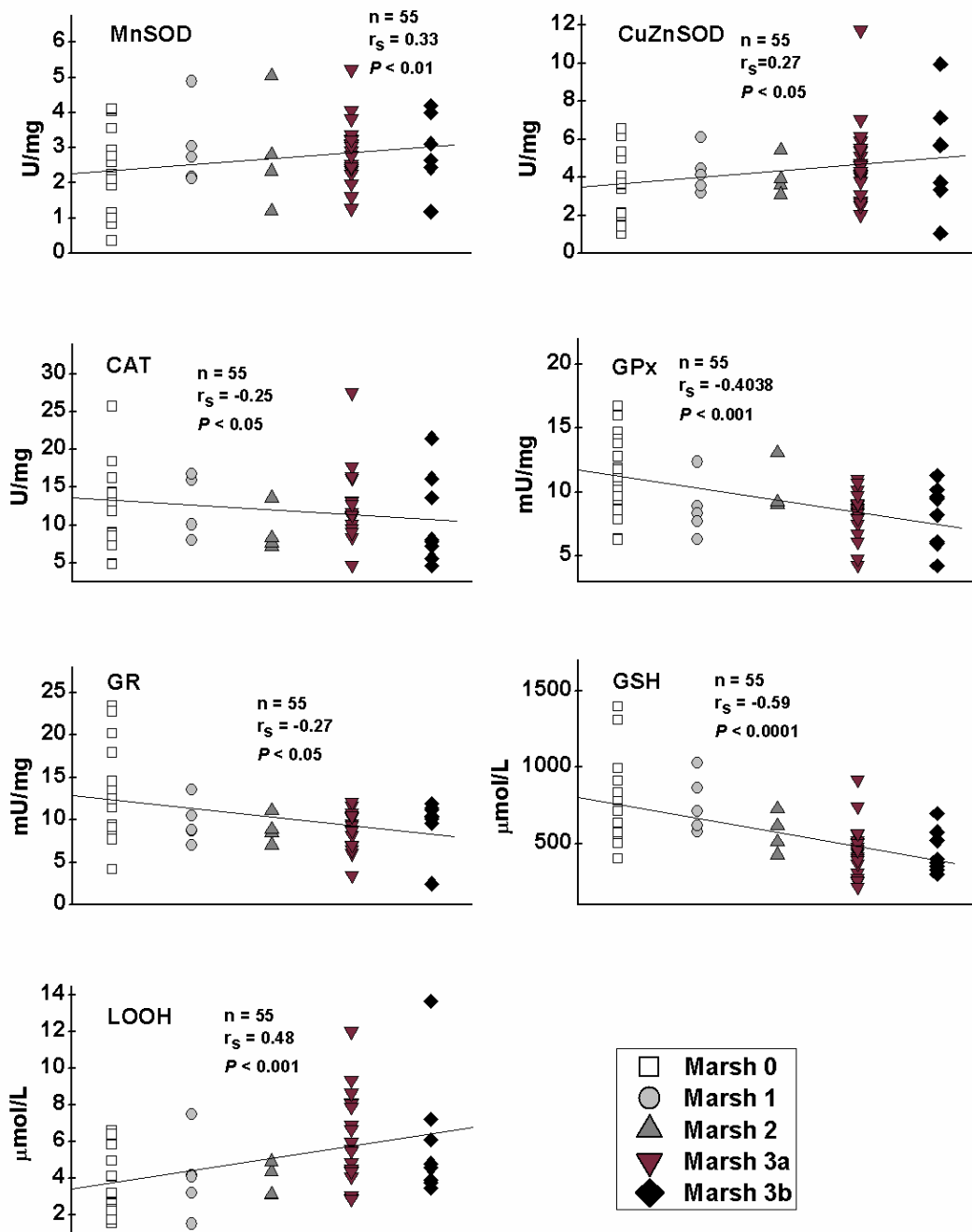


Fig. 3. Data plot and coefficients of Spearman's rank correlation r_s between the parameters of antioxidant status and the severity of mucosal lesion in celiac patients. MnSOD - manganese superoxide dismutase, CuZnSOD - copper-zink superoxid dismutase, CAT - catalase, GPx - glutathione peroxidase, GR - glutathione reductase, GSH - glutathione, LOOH - lipid hydroperoxides; Marsh 0 - normal intestinal mucosa, Marsh 1 - mucosa with intraepithelial lymphocytosis, Marsh 2 - mucosa with intraepithelial lymphocytosis accompanied by crypt hyperplasia, Marsh 3a - mucosa with partial villous atrophy, Marsh 3b - mucosa with subtotal villous atrophy.

4. Discussion

There is a growing body of evidence showing that ROS are involved in the pathology of celiac disease. Pro-oxidant effects of gliadin have also been reported in celiac patients. Activation of XO is one of the mechanisms of free radical and ROS overproduction in the small intestinal mucosa. The xanthine oxidoreductase system is mainly located in the intestinal mucosa and liver (Sarnesto et al., 1996). Distribution of this enzyme in the small bowel is not uniform. Histological studies have shown that the main part of XO is located in the epithelial cells at the top of the intestinal villi, while no XO activity could be detected at the basis of crypts (Pickett et al., 1970). The results of Boda and coworkers (1999) suggest that in patients with active celiac disease, gluten ingestion, along with the resulting inflammation, causes activation of XO in enterocytes, which results in overproduction of ROS and further damage to the mucosa. These pro-oxidant processes are counteracted by AO enzymes MnSOD and CuZnSOD. The activity of MnSOD in our study was elevated in patients with villous atrophy comparing to the Marsh 0 group, while CuZnSOD activity did not vary significantly. However, positive correlations between the activities of both SODs and the degree of mucosal lesion indicate that more severe mucosal damage is associated with increased enzyme activities. This may represent a physiological response to a higher rate of ROS production. On the contrary, the activity of CAT did not vary significantly between the analyzed groups and it correlated inversely with the mucosal lesion. This may be a consequence of the kinetic characteristics of this enzyme. Namely, due to its high Michaelis-Menten constant, CAT is most efficient against high H_2O_2 concentrations. When H_2O_2 concentrations are lower, more effective protection is given by GPx, another H_2O_2 detoxifying enzyme (Eaton, 1991). Since the activity of GPx, the main scavenger of H_2O_2 in gastro-intestinal tissue was significantly decreased in patients with villous atrophy (Marsh 3a and Marsh 3b), the imbalance between H_2O_2 production and scavenging causes pro-oxidant shift, which results in increased LPO and LOOH concentration. In these patients LOOH concentration was ~ 80 % higher than in patients with normal mucosa. Even in some GFD patients (Marsh 1+2) LOOH concentration was elevated ~ 20 % in comparison to the Marsh 0 group. These results are in accordance with the data from an *in vitro* study (Rivabene et al., 1999) where the concentration of LOOH in cell culture was 30-50 % higher after gliadin treatment.

Increased LOOH levels may contribute to the disruption of detoxifying pathways in the bowel and to dysfunction of enterocytes, which may cause various disorders of the digestive tract (Aw, 1998). The intestine differs from other fully differentiated organs by a very high rate of cellular turn over. The lifespan of enterocytes is only 4-6 days (Iatropoulos, 1986). Due to the high cell division rate, the chance of spreading a mutation to the subsequent cell generations is much higher than in cells with a low division rate, which makes the intestine very susceptible to mutagenesis and cancerogenesis. It has been shown that subtoxic concentrations of LOOH can provoke a change in the cellular redox state enough to enhance a mitogenic response in rat enterocytes (Aw, 1999), while more severe oxidative stress activates pro-apoptotic processes (Imai & Nakagawa, 2003). Several *in vitro* studies have also demonstrated that exposure of human intestinal cells to the subtoxic concentrations of LOOH can induce cell transition from a quiescent to a proliferative state or even growth arrest (Gotoh et al., 2002), while high LOOH levels disturb the intestinal homeostasis to such an extent that pro-apoptotic processes cannot be stopped even after restoration of redox balance (Wang et al., 2000). In addition, LOOH cause damage to intestinal cell membranes in

vitro, as well as single- and double-strand DNA breaks and may influence the activity of AO enzymes (Wijeratne & Cuppett, 2006).

Since the intestinal mucosa interfaces with the lumen, which is open to the exterior environment and deeper layers of the intestinal wall, it represents a crucial protective barrier against potential toxic agents. In addition to the nutrients, the intestinal mucosa is constantly exposed to oxidants, mutagens and carcinogens from the diet, as well as to endogenous ROS. Several protective mechanisms preserve cellular integrity and tissue homeostasis: the intestine is able to maintain high concentrations of antioxidants and to up-regulate AO enzymes, while apoptosis is induced to eliminate spent or damaged enterocytes (Aw, 1999). The GSH redox cycle is the key mechanism of LOOH scavenging in the intestine (Aw, 2005). GSH is a powerful antioxidant that acts as a detoxifier of endogenous and exogenous ROS. Published data indicate that epithelial cells of the small and large intestine are highly dependent on GSH. It was demonstrated that in mice treated with L-buthionine SR-sulfoximine (BSO), a specific inhibitor of GSH synthesis, a significant degeneration of enterocytes is induced, as a consequence of GSH deficiency (Mårtensson et al., 1990). High levels of GSH are found in many tissues, including the intestine. Normal concentrations of intracellular GSH are maintained by *de novo* synthesis, regeneration from GSSG or through import via the Na⁺-dependent transport system. GSH transport into the cell is demonstrated in several cell types, including enterocytes (Aw, 1994; Mårtensson et al., 1990). The ability of enterocytes to import luminal GSH is important for the intestinal thiol balance, especially in pro-oxidative conditions, since the human diet is extremely various concerning the GSH and LOOH content. In a healthy system, where GSH is not limiting, intracellular metabolism of LOOH is enhanced, decreasing luminal LOOH retention and excretion into lymph. On the contrary, if GSH is insufficient, LOOH catabolism decreases and their luminal retention and lymphatic transport are promoted (LeGrand and Aw, 2001).

In this investigation a significantly lower GSH concentration (~ 40-50 %) was found in the intestinal mucosa with villous atrophy compared to the normal mucosa and mucosa with milder lesions. The decreased GSH concentration is followed by decreased activities of GPx and GR and increased LOOH concentration. Our results are in accordance with the previous data reporting a significant decrease of GPx activity in mucosa of children with severe villous atrophy (Ståhlberg et al., 1988). Similar data have come from *in vitro* studies investigating the effects of gliadin on intestinal cells in culture (Dolfini et al., 2002; Dolfini et al., 2005). The experiments of Rivabene and coworkers have shown that the antiproliferative effects of gliadin are associated with pro-oxidative changes in the cell, such as elevated LOOH levels, decreased GSH concentration and a loss of SH- groups in proteins; the administration of BSO has demonstrated that the extent of these changes depended on the basal redox state of enterocytes, primarily their GSH content (Rivabene et al., 1999). These results imply that higher GSH concentrations could, at least partly, modify cell susceptibility to the toxic effects of gliadin.

GSH is not only an enzyme cofactor, but can also react directly with free radicals and is involved in recycling of other chain breaking antioxidants, such as vitamin E, whose concentration is reduced in celiac patients on GFD (Odetti et al., 1998). One of the extremely important roles of GSH is detoxification of various endogenous and exogenous toxins by GSH-S-transferases (GST), which use GSH as a cofactor. GSH deficiency should also influence these enzymes. Previous investigations have reported reduced total GST activity,

as well as GST α class levels, although GSH concentration was not significantly altered in comparison to the control values (Wahab et al., 2001). In an in vitro study gliadin provoked a reduction in GSH content and gliadin concentration-related decrease in the activity of GPx, GR and GSH-S-transferases (Elli et al., 2003).

Since the activities of GPx and GR in our study were inversely related to GSH concentration, a decrease in these activities in patients with villous atrophy could be a consequence of GSH deficiency. In addition, as Gpx is a selenium-dependent enzyme, low Se levels can also influence its activity. Previous investigations have shown that GPx activity did not change in the conditions of elevated LOOH levels, pointing to the influence of dietary selenium on the glutathione peroxidase system of the gastrointestinal tract in rats (Reddy & Tappel, 1974; Vilas et al., 1976). Selenium deficiency has already been reported in celiac patients (Stazi & Trinti, 2010; Yuce et al., 2004). Several studies have also reported a strong correlation between Gpx activity and Se level in the blood, especially when Se concentration is low ($< 80 \mu\text{g/L}$) (Lloyd et al., 1989; McKenzie et al., 1978). Aw and coworkers suggested that GPx and GR *per se* are not limited in the catabolism of LOOH in the intestine. The kinetics of these enzymes and the extent of intracellular LOOH degradation depend principally on the availability of cellular reductants (GSH and NADPH) (Aw & Williams, 1992; Aw et al., 1992). It has been shown that a disturbed intestinal GSH/GSSG ratio is involved in the pathogenesis of chronic intestinal inflammation in mice with severe combined immunodeficiency (SCID) reconstituted with CD4⁺CD45Rb^{high} T-lymphocytes (Aw, 2005). The loss of GSH redox balance preceded tissue hyperplasia, mucosal inflammation and the symptoms of clinical colitis, suggesting that redox imbalance may be a contributor rather than the consequence of disease.

Similar to previous reports, the results of our study indicate that oxidative stress is strongly associated with celiac disease and could be an important factor in disease pathogenesis. The AO status of patients with villous atrophy is severely disturbed. GSH deficiency and decreased activities of GSH-dependent enzymes significantly reduce the AO capacity of these patients, which is reflected in elevated LOOH levels. The AO status of patients with milder mucosal lesions (Marsh 1+2 group) was not significantly altered compared to patients with normal mucosa. However, it should be emphasized that even in this group LOOH concentration was significantly elevated pointing out the incapacity of the GSH redox cycle to efficiently eliminate peroxides. The LOOH challenge may provoke perturbations of normal intestinal cell proliferation, differentiation and apoptotic responses, contributing to the increased risk of malignancy in untreated celiac patients. It is well known that GSH is indispensable for normal function of intestinal epithelium. Since enterocytes are capable of absorbing dietary GSH from the intestinal lumen, it has been demonstrated that oral administration of GSH elevates GSH concentration in small and large intestinal mucosa of mice, protecting the tissue from GSH deficiency provoked by BSO (Mårtensson et al., 1990). We could speculate that the oral administration of GSH would be beneficial for protection of the intestinal epithelium from damage occurring in inflammatory disorders, such as celiac disease, as well as ischemia, chemotherapy or radiation. In addition, as GSH can be regenerated from other antioxidants (Lenton *et al.*, 2003), a diet enriched with natural antioxidants, as well as appropriate supplements, could be important complements to the classic GFD. Furthermore, since GPx is more susceptible

to the influence of dietary supplements than other AO enzymes (Andersen et al., 1997), administration of antioxidants and Se could enhance GPx activity and promote LOOH scavenging in celiac patients.

Previous data concerning correlations between the activities of AO enzymes and histological changes characteristic of celiac disease are limited. Wahab and coworkers (2001) found decreased levels and activities of GST in small intestinal mucosa of celiac patients in comparison to the healthy individuals, which were proportional to the degree of mucosal damage. Similarly, the activity of uridine 5'-diphospho-glucuronosyltransferase, an enzyme that detoxifies some noxious compounds by catalyzing their addition to glucuronic acid, was also reduced in the intestinal mucosa of patients with celiac disease (Goerres et al., 2006). On the contrary, plasma concentrations of end products of NO metabolism in celiac patients were elevated and correlated with the histological changes of mucosa (Murray et al., 2003; Spencer et al., 2004). The results of our study show that the severity of the mucosal lesion in celiac patients significantly correlated with all analyzed parameters of AO status. Positive correlations were found between severity of histological damage and the activities of both SODs, as well as LOOH concentration. The activities of CAT, GPx and GR and the concentration of GSH inversely correlated with the degree of the mucosal lesion. Concerning the fact that the severity of histological damage is reflective of the AO status in the intestinal mucosa of celiac patients and since similar changes of the AO status in peripheral blood have already been reported (Stojiljković et al., 2007), we suggest that new, noninvasive methods, involving analysis of AO parameters, could be developed for prediction and follow-up of histological changes in patients with celiac disease. In addition, nutritional evaluation should be performed in celiac patients at least annually, to monitor the mucosal damage and the effectiveness of GFD.

5. Conclusions

Our results demonstrate that oxidative stress is implicated in the pathogenesis of celiac disease. A significant disturbance in AO status occurs in patients affected by celiac disease, especially those with the advanced mucosal damage. Changes of AO status significantly correlate with the severity of histological damage. A seriously impaired AO capacity for degradation of lipid hydroperoxides may persist even after several years of GFD. A diet rich in natural antioxidants as well as appropriate dietary supplements could be beneficial for full mucosal healing of celiac patients

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