

CLINICAL STUDY

The significance of increased levels of end nitric oxide metabolites in blood serum of children with celiac disease

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Abstract: *Objectives:* The aim of the study was to determine serum nitric oxide (NO) metabolites and correlate them to gluten-free diet (GFD) compliance, the parameters of morphological and functional condition of the small intestine in patients with celiac disease (CD) in a long-term follow-up.

Patients and methods: The study included 35 children (age median 8.0 years) with previously diagnosed CD, among them 15 – on a strict GFD and 20 – on a semistrict GFD. 32 patients (age median 7.5 years) with functional abdominal pain were recruited as controls. Nitrites (NO₂), nitrates (NO₃), total content of nitrites and nitrates (NOx) in serum were determined using the Griess reaction. CD patients underwent small intestine morphometry and absorption D-xylose test.

Results: Compared to the controls, the CD patients on a strict GFD had no significant differences in NO₂, NO₃, NOx content, while in patients violating GFD these parameters were significantly higher. In comparison with the patients on a strict GFD, the patients on a semistrict GFD had higher median values of NO₃ (p = 0.002) and NOx (p = 0.0000). Serum NOx correlated negatively with the height of villi (p = 0.0001), D-xylose excretion (p = 0.0063), and correlated positively with the depth of crypts (p = 0.0082) and cellular density of villi stroma (p = 0.0067).

Conclusion: CD patients not keeping to a strict GFD have an increased level of nitric oxide metabolites in serum. NOx content is associated with morphometric parameters of the small intestine that proves the participation of NO in the pathogenesis of histological changes in CD (Tab. 3, Ref. 39). Text in PDF www.elis.sk.

Key words: celiac disease, follow-up, nitric oxide, nitrite, nitrate.

Celiac disease (CD) is chronic, immunologically determined form of enteropathy affecting the small intestine in genetically predisposed children and adults (1). At present, epidemiological studies in different countries indicate significant prevalence rate of the pathology among the population from 0.3 % to 2 % (2–4). The highest risk of the disease is associated with the presence of genetic markers of HLA-DQ2 and HLA-DQ8 molecules. CD is provoked by intake of wheat, rye, barley products, which contain gluten and gluten-related gramineous proteins. In CD, gluten has toxic effect on small intestine promoting an immuno-inflammatory process in intestinal mucosa and secreting proinflammatory cytokines that can result in atrophy of intestinal villi, malabsorption, and various clinical presentations (5–7).

Inflammation is known to be associated with the production of nitric oxide (NO) and its transmutation products (8, 9). Duodenal mucosa of CD patients was found to have significant increase of

activity of inducible nitric oxide synthase (iNOS) that results in increased production of NO and total level of stable end-products of its oxidation (NOx), i.e., nitrites (NO₂) and nitrates (NO₃) (10, 11).

The increased level of nitric oxide metabolites was found in urine of children with CD (12), and proved by other authors as well (13). High content of nitric oxide metabolites was also found in plasma and serum of untreated CD patients (14, 15). A follow-up for several months after diagnosis verification and treatment administration in CD patients showed a decreased level of plasma nitric oxide products (16).

A life-long strict gluten-free diet (GFD) is recognized to be the only treatment modality of CD and prevention of its complications (17). However, due to various reasons, not all patients adhere to a strict GFD after diagnosis of CD (18, 19).

Potential clinical value of the measurement of nitric oxide products in blood serum of CD patients in long-term follow-up after diagnosis is understudied.

In our view, the solution of the problem by means of minimally invasive technique will enable to control GFD observation in CD patients and the dynamics of the process in the small intestine; and contribute to the extension of an idea of NO involvement in mechanisms participating in the change of the structure of intestinal mucosa in CD.

The objective of the present study was to assess the level of nitric oxide metabolites in serum of children with CD in a long-term follow-up, and to investigate a possible relationship between the level of nitric oxide metabolites and gluten-free diet compli-

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ance, the indices of morphological and functional condition of intestinal mucosa.

Patients and methods

We examined 35 children including 10 boys and 25 girls (median age 8.0 years) with classical celiac disease 1.5–14.5 years after CD diagnosis based on revised criteria of the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (20).

Special interviewing showed that 15 (42.9%) out of 35 patients kept to a strict GFD, and 20 (57.1%) children partially violated the diet (semistrict GFD).

Inclusion criteria for patients were the following: age from 3 to 17 years, follow-up period from 1.5 years and longer after diagnosis verification, the confirmation of the diagnosis of CD according to previous serological and histological examination prior to GFD prescription, and patient Informed Consent. Exclusion criteria were: age under 3 and over 17 years, a follow-up period less than 1.5 years, untreated CD, the presence of concomitant acute respiratory disease, the lack of an informed consent of patients and their parents.

Control group included 32 children with functional abdominal pain: 12 boys and 20 girls (median age 7.5 years). Patients of both groups (CD group and control group) were comparable by age ($p = 0.6915$) and sex ($p = 0.6031$).

Blood samples for biochemical study were taken in the morning, after at least 8-hour fasting period, by cubital vein puncture. Serum was received by standard methods. Serum samples were drawn in all examined patients to determine nitric oxide metabolites and other biochemical parameters. Total nitrites and nitrates and nitric oxide content were evaluated by the Greiss reaction,

and nitrates were reduced into nitrites using vanadium chloride. We took nitrite content from total nitric oxide metabolites to determine nitrate concentration. The sum of nitrites and nitrates was measured as an indirect variable of NO production. The results of the analysis were recorded using microplate photometer Multiskan Ascent (Labsystems Oy, Finland) with wavelength of 540 nm (21, 22).

Hemoglobin concentration was measured by hematological analyzer ABX Pentra 60 (HORIBA ABX, France). Serum albumin level was measured using automatic biochemical analyzer CA-180 (FURUNO, Japan).

All patients with CD underwent gastrointestinal endoscopy with target biopsy of mucosa from distal duodenum. The samples were fixed in 10% neutral formalin, and then embedded in paraffin, cut 5 μm thick, stained by hematoxylin and eosin for the following light microscopy and morphometry. The samples were studied by 2 pathologists with long-term experience in morphometric analysis, who did not know the research topic. Well-oriented biopsy samples were studied by the length of villi, the depth of crypts, the number of intraepithelial lymphocytes (per 100 enterocytes), as well as total cellular density in a square millimeter of lamina propria villi and the space between the crypts using a microscope Nikon Eclipse E400, and quantitative morphometric indices of intestinal mucosa condition were obtained.

To assess intestinal absorption in CD patients we used urinary D-xylose test performed according to standard technique, calculating a fraction of xylose dose excreted in 5 hours in urine. The findings of urinary D-xylose test were expressed in %.

The research was in compliance with local biomedical ethics committee requirements of Nizhny Novgorod Federal Research Institute of Pediatric Gastroenterology developed according to

Tab. 1. Demographic and laboratory parameters of subjects.

Parameters	Control group n=32	Total CD patients n=35	CD patients		p
			Strict GFD n=15	Semistrict GFD n=20	
Age, years	7.5 (5.75–12.0)	8.0 (6.0–11.0)	8.0 (6.0–10.5)	9.5 (6.0–13.0)	0.6915
Sex (male/female)	12/20	10/25	3/12	7/13	0.6031
Blood hemoglobin, g/l	134.0 (122.0–140.5)	131.0 (122.5–139.3)	130.0 (123.0–138.0)	134 (125.0–138.5)	0.5949
Serum albumin, g/l	44.25 (40.75–45.90)	43.85 (41.50–46.98)	44.50 (40.90–47.48)	43.55 (42.00–46.53)	0.5096
Villi height, μm	–	385.0 (308.0–427.0)	441.0 (406.0–456.5)	329.0 (285.3–368.0)	0.0000
Crypt depth, μm	–	170.0 (156.0–180.5)	163.0 (156.0–166.5)	173.5 (163.0–228.0)	0.0180
IEL/100 villi enterocytes	–	19.0 (16.5–24.0)	19.0 (17.0–21.0)	21.0 (16.0–26.5)	0.4819
Cell density intravillus	–	9709.0 (9238.0–10593.5)	9238.0 (9097.0–9568.0)	10275.0 (9521.0–10982.3)	0.0092
Cell density in the space between crypts	–	10558.0 (9362.0–11123.0)	9804.0 (9144.0–10511.0)	10841.0 (10150.3–11383.0)	0.0170
D-xylose % of dose/5 h	–	28.85 (25.38–34.55)	32.29 (28.68–40.25)	26.80 (21.70–29.63)	0.0185

CD – celiac disease, IEL – intraepithelial lymphocytes.

Cell density is expressed as the number of total cells per 1 mm² of lamina propria. Data are presented as median (interquartile range) unless otherwise indicated, and calculated using the Mann-Whitney U test for continuous variables, and Fisher's exact test for categorical variables.

Tab. 2. The level of end products of nitric oxide metabolites in serum of patients with celiac disease (CD) and control group.

Parameters	Control group n=32 (1)	Total CD patients n=35 (2)	CD patients		P value (U-test)
			Strict GFD n=15 (3)	Semistrict GFD n=20 (4)	
NO ₂ , µmol/l p value 0.0280 (K-W)	12.35 (8.40-15.48)	14.60 (9.53-22.95)	11.70 (9.33-18.10)	18.40 (10.55-28.80)	p1-2=0.0333 p1-3=0.4651 p1-4=0.0099 p3-4=0.0890
NO ₃ , µmol/l p value 0.0005 (K-W)	17.20 (10.30-30.03)	29.10 (17.90-54.65)	21.30 (10.10-26.95)	44.75 (30.60-72.63)	p1-2=0.0192 p1-3=0.9091 p1-4=0.0003 p3-4=0.0020
NO _x , µmol/l p value 0.0000 (K-W)	31.25 (23.08-40.23)	54.90 (35.40-69.70)	35.10 (26.25-37.25)	66.45 (56.63-93.00)	p1-2=0.0002 p1-3=0.7064 p1-4=0.0000 p3-4=0.0000

NO₂ – nitrite, NO₃ – nitrate, NO_x – nitrite/nitrate. Data are presented as median (interquartile range) unless otherwise indicated. K-W Kruskal-Wallis H-test, U Mann-Whitney U test, p – level of significance, p_{1,2} – statistically significant differences between group 1 and 2, p_{1,3} – statistically significant differences between group 1 and 3, p_{1,4} – statistically significant differences between group 1 and 4, p_{3,4} – statistically significant differences between group 3 and 4.

World Medical Association's Declaration of Helsinki, and a signed informed consent was obtained from each patient and his parents.

Statistical analysis was performed using software environment for statistical computing R, version 2.15 (23).

Shapiro–Wilk test was used to control the normality of distribution of the indices. We used Levene test for homogeneity of variances. Since Gauss distribution and homogeneity of variances were not confirmed, nonparametric techniques were applied. Continuous variables were represented as medians (Me) and interquartile intervals and analyzed using Kruskal–Wallis H-test and Mann–Whitney U test. Categorical variables were expressed as counts and analyzed using Fisher's exact test. The correlations between NO_x and other laboratory parameters were studied by Spearman's coefficient. The differences were considered statistically significant at a significance level of $p < 0.05$.

Results

Table 1 represents demographic and laboratory parameters of control children and CD patients (total CD, CD on a strict GFD, CD on a semistrict GFD). The comparison of routine laboratory parameters between the general group of CD patients and the control group did not reveal any statistically significant differences in hemoglobin level ($p = 0.6915$) and serum albumin level ($p = 0.5096$). There were no significant differences in hemoglobin and albumin levels between the control patients and those with CD on a strict GFD, and between the control patients and those with CD on a semistrict GFD.

The mucosal condition examined by morphometric findings differed in CD patients on a strict GFD and those on a semistrict GFD. Compared to the patients keeping to a strict GFD, in patients violating a strict GFD the height of villi was lower ($p = 0.0000$), and it was larger than crypt depth value ($p = 0.0180$), cellular density of lamina propria in villi ($p = 0.0092$) and the space between the crypts ($p = 0.0170$). Urinary D-xylose excretion was higher in patients on a strict GFD than in those on a semistrict GFD ($p = 0.0185$).

Table 2 demonstrates the investigation results of the end products of nitric oxide metabolites. The comparison of the two groups: total CD patients and control children showed the group of total CD patients to have higher values of NO₂ ($p = 0.0333$), NO₃ ($p = 0.0192$), and their total content: NO_x in serum ($p = 0.0002$). The assessment of the parameters of products of nitric oxide metabolites using Kruskal–Wallis H-test revealed significant differences between the three groups: controls, CD patients on a strict GFD, and patients on a semistrict GFD by median values NO₂ ($p = 0.0280$), NO₃ ($p = 0.0005$) and NO_x ($p = 0.0000$).

In comparison with the control group CD patients on a strict GFD had no significant differences in content of NO₂ ($p = 0.4651$), NO₃ ($p = 0.9091$), NO_x ($p = 0.7064$), while CD patients on a semistrict GFD compared to controls had statistically significantly higher level of serum NO₂ ($p = 0.0099$), NO₃ ($p = 0.0003$), NO_x ($p = 0.0000$). As compared to the children on a strict GFD, those on a semistrict GFD showed higher median values of NO₃ (21.30 µmol/l versus 44.75 µmol/l; $p = 0.0020$) and NO_x (35.10 µmol/l versus 66.45 µmol/l; $p = 0.0000$).

Table 3 shows the correlation analysis. Spearman rank correlation analysis performed in all CD patients revealed negative correlation between NO_x concentration and the height of duodenal mucosal villi ($p = 0.0001$) and the value of absorption test with

Tab. 3. Correlation analysis between serum NO_x level and other laboratory parameters in celiac disease (n = 35).

	General correlations	
	Spearman's Rho	p
Villi height	–0.59	0.0001
Crypt depth	0.44	0.0082
IEL	0.13	0.4415
Cell density intravillus lamina propria	0.45	0.0067
Cell density of lamina propria in the space between crypts	0.35	0.0390
D-xylose excretion	–0.51	0.0063

IEL – intraepithelial lymphocytes

urinary D-xylose ($p = 0.0063$). NOx level correlated positively with the depth of crypts ($p = 0.0082$), total cellular density of lamina propria villi ($p = 0.0067$) and total cellular density of lamina propria of the space between the crypts ($p = 0.0390$). There was no correlation between NOx level and intraepithelial lymphocyte count ($p = 0.4415$).

Discussion

Currently, there is a great demand for the development of tests useful in the monitoring of CD patients in a long-term follow-up period that is of primary importance for the disease outcome and has an effect on health care system costs (24).

The aim of the present study was to determine the levels of nitric oxide metabolites in serum and reveal possible relationship between them and gluten-free diet compliance, indices of morphological and functional condition of intestinal mucosa in children with CD in a long-term follow-up.

The present study found that compared to control group, the levels of NO oxidates (i.e. nitrites, nitrates and their total content) in serum of patients on a strict GFD had no statistical differences, while in CD patients keeping to a semistrict GFD these indices were significantly higher. NO₃ and NOx concentrations in patients on a semistrict GFD were statistically significantly higher than in patients keeping on a strict GFD.

The data on the level of nitric oxide products in CD patients keeping to a strict GFD correspond to those reported by Ertekin et al (15). However, the study of Hollen et al (25) reports that a part of CD patients keeping to a traditional GFD have not normalized their nitrites/nitrates after a one-year diet.

Our study has found that the breach of diet compliance in CD is accompanied by an increase of the level of nitric oxide metabolites in blood serum. It seems that an increased concentration of nitric oxide products in serum in CD can indicate the intensity of an inflammatory process in the small intestine. Documentary evidence of the supposition is the revealed relationship between NOx and total cellular density of lamina propria villi and the space between the crypts in the group of total CD patients. Moreover, it is supported by the fact we have revealed: significantly higher cellular density throughout the depth of duodenal lamina propria mucosa in children with the breach of GFD compared to those on a strict GFD. The continuing intake of gluten products in patients with diagnosed CD is supposed to result in steady inflammatory process in intestinal mucosa (26). In addition, we have found significant negative correlation between NOx concentration and villi height, and positive correlation between NOx and crypt depth in children with CD. The revealed reduced height of villi and the increase of crypt depth in breach of GFD compared to a strict GFD is an evidence of a damage effect of gluten on intestinal mucosa in CD patients. According to literature reports, the damage of villi is caused by the presence of a significant accumulation of inflammatory cells in the upper part of lamina propria (27). Our findings on the correlations between nitric oxide metabolites and histological condition of intestinal mucosa in CD patients in follow-up differ from those obtained in the previous study. The latter reported of

plasma NOx to be correlated with histological grade only initially (16). It may be explained by the way the small intestine biopsies are assessed. We share the opinion that only quantitative results of mucosal parameters obtained morphometrically, especially in a follow-up period, is the most sensitive and objective way to record the changes in the small intestine biopsies (28, 29) that enables to draw a significant comparison between them and other laboratory findings of a particular patient.

Our findings have revealed that serum NOx level in CD patients correlates negatively with the index of urinary D-xylose excretion within 5 hours that is widely used as an absorption test (30).

The study has showed that the increase in the concentration of nitric oxide metabolites in serum reflects abnormal morphology of the small intestine and its reduced absorption function in patients with the breach of a strict GFD. An increased concentration of end products of nitric metabolites in serum in children with CD on a semistrict GFD may also indicate high NO production.

By now, NO in body is known to be synthesized from the amino acid L-arginine by three known isoforms of NO-synthase enzyme: neuronal (nNOS), endothelial (eNOS) and inducible (iNOS). The latter synthase provides a long-standing synthesis of the greater number of NO than other isoforms. iNOS promotes the pathophysiology of inflammatory diseases (31). In gastrointestinal tract iNOS is activated in many gastrointestinal diseases. In addition, NO and its products can have protective or tissue damaging effect (32–34).

The previous researches in intestinal biopsies in CD showed that in enterocytes and lamina propria of mucus there is an elevated expression of iNOS, the source of which are inflammatory cells (11, 35). Therefore, the increase in the level of nitric oxide metabolites in biological media in CD patients is certain to be the effect of enhanced iNOS action of the bowel.

In a number of researches there has been shown that interferon-gamma in combination with gliadin can contribute to the elevated expression of iNOS genes in intestinal macrophages in celiac disease (36, 37) that increases nitric oxide production. Bernardo et al (38) in their work found that in CD patients after the production startup of nitrites and interferon-gamma interleukin-15 induces more intense immune response that results in the production of other immune mediators and increases intestinal damage. It should be admitted that many aspects of the participation of iNOS, NO and its derivatives in the pathogenesis of immuno-inflammatory process in celiac disease are still not discovered completely.

Food intake of nitrates, the biological half-life of which is 5–8 h can have an impact on the level of NO metabolites in blood serum (39), and therefore, a fasting period before taking blood serum samples is recommended. In our study the blood serum samples were taken fasting in the morning after nocturnal sleep. Significant differences in the content of nitrates and nitrites between the groups of children with CD (a strict GFD and a semistrict GFD) revealed by our research are unlikely to be explained by other dietary factors but gluten.

The limitation of the method for determination of the end products of nitric oxide in serum is the impossibility to study opaque blood serum. However, in clinical practice such cases are rare.

Conclusion

The level of stable nitric oxide end products in serum in children with CD breaking a strict GFD in a long-term follow-up period is significantly increased. The changes of serum NOx content in CD patients are significantly associated with particular morphometrical parameters of intestinal mucosa and the value of a functional absorption test. The increase of the level of nitric oxide metabolites accompanies the exacerbation of chronic inflammatory process with villi damage in the intestinal mucosa in children breaking a strict gluten-free diet. The concentration of nitric oxide metabolites in blood serum is a useful, minimally invasive and quick measured biomarker to monitor dietary compliance and the intestinal mucosa condition in children with CD in annual examination in clinic. Further studies will enable to improve the system of a long-term follow-up in CD patients and provide up-to-date information about NO participation in the pathogenesis of the disease.

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