

Elevated Nitrosative Stress in Children with Chronic Spontaneous Urticaria

Fatih Dilek^a Deniz Ozceker^c Emin Ozkaya^a Eray Metin Guler^b
Mebrure Yazici^a Zeynep Tamay^c Abdurrahim Kocyigit^b Nermin Guler^d

Departments of ^aPediatric Allergy and Immunology and ^bClinical Biochemistry, Bezmialem Vakif University Medical Faculty, ^cDepartment of Pediatric Allergy, Istanbul Medical Faculty, Istanbul University, and ^dDepartment of Pediatrics, Istanbul Bilim University, Istanbul, Turkey

Keywords

Chronic spontaneous urticaria · Chronic urticaria · Nitrosative stress · Nitric oxide · Nitrite · Nitrate · Children · Disease severity

Abstract

Background: Chronic spontaneous urticaria (CSU) is an idiopathic condition that seriously affects quality of life. It is well known that oxidative stress and nitrosative stress (NS) are generally involved in many chronic inflammatory diseases. This study aimed to evaluate the possible role of NS in the pathogenesis of CSU. **Methods:** Thirty-two children with CSU and 22 healthy control subjects were enrolled in the study. Demographic and clinical features were defined, and disease activity was quantified using the urticaria activity score (UAS). Serum NS was assessed by the plasma levels of total nitric oxide (NOx) metabolites and nitrite and nitrate measurements using a Griess method-based commercial kit. **Results:** Plasma NOx levels were 82.5 ± 11.3 $\mu\text{mol/L}$ in the CSU group and 50.9 ± 9.4 $\mu\text{mol/L}$ in the control group. The difference was statistically significant ($p < 0.001$). CSU patients also had higher plasma nitrite levels than controls (53.3 ± 13.8 vs. 30.2 ± 10.1 $\mu\text{mol/L}$, respectively, $p < 0.001$). The median values of plasma nitrate were 27.5 $\mu\text{mol/L}$ (IQR

19.1–35.5) in CSU patients and 20.9 $\mu\text{mol/L}$ (IQR 17.9–23.2) in the control group, and the difference was statistically significant ($p = 0.009$). In addition, plasma NOx and nitrite levels were positively correlated with the UAS ($\rho = 0.512$, $p = 0.03$ and $\rho = 0.452$, $p = 0.011$, respectively). **Conclusion:** Plasma NS is elevated and positively correlated with disease activity in children with CSU.

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Introduction

Chronic urticaria (CU) is defined as the presence of a urticarial episode with or without angioedema for at least 6 weeks [1]. It is a debilitating disease and seriously affects quality of life [2]. The disease has 2 subtypes, namely chronic inducible urticaria (CIU) and chronic spontaneous urticaria (CSU) [2]. If an urticarial episode appears following a physical stimulus like pressure, heat, or cold, this subtype of the disease is referred to as CIU. In contrast, if wheals, angioedema, or both appear without an identified stimulus, this subtype of CU is considered CSU [2].

Although it is well known that CU is an inflammatory disease and mast cell activation is the major feature of CU

pathogenesis, the underlying factors of this phenomenon are not yet completely understood [1]. During the course of inflammation, activation of oxidant-generating enzymes, such as myeloperoxidase, NADPH oxidase, and inducible nitric oxide synthase (iNOS) lead to the production of excess amounts of reactive oxygen and nitrogen species [3]. In addition, reactive oxygen species derived from NADPH oxidases further increase the expression of iNOS [4]. Overproduction of these reactive molecules can disrupt the balance between oxidant and antioxidant systems and cause oxidative stress (OS) and nitrosative stress (NS) [3].

Nitric oxide (NO) is the primary source and initiator of NS [5, 6]. Although the most familiar biological effect of NO is vasodilatation, NO also plays critical regulatory roles in many other processes in the human body, such as gene transcription, neurotransmission, wound healing, apoptosis, immunity, and inflammation [5, 6]. Elevated NO levels have been reported in various diseases, such as rheumatoid arthritis, septic shock, cancer, and many cardiovascular and neurodegenerative diseases, as well as allergic diseases, such as asthma, allergic rhinitis, and atopic dermatitis [5, 7–9]. OS and NS are inextricably intertwined in metabolic processes of the human body, as well as in the pathogenesis of allergic diseases, including asthma and atopic dermatitis [8, 10–13]. However, according to our literature search, the possible role of NS in the pathogenesis of CSU has not yet been evaluated. Thus, we aimed to clarify this issue in the present study.

Materials and Methods

Participants

This study was designed using a cross-sectional format, and it was carried out between July 2015 and April 2016 in the outpatient clinics of 2 university hospitals (Bezmialem Vakif University and Istanbul University/Istanbul Medical Faculty). A total of 49 pediatric CU patients who were admitted to 1 of these centers in the last year were invited to participate in the study. Patients who were diagnosed with CIU and those for whom the potential causes of CU had been defined (except autoimmunity) were excluded from study. Diagnosis was based on the European Academy of Allergy and Clinical Immunology (EAACI) guidelines [2].

None of the patients were taking immunosuppressive drugs, corticosteroids, or omalizumab. Detailed medical histories were obtained and physical examinations performed. Children with diseases that could potentially influence plasma OS and NS levels, including concomitant diagnosed asthma, allergic rhinitis, atopic dermatitis, and any other chronic disease, as well as children taking multivitamin, mineral, or dietary supplements, were also excluded from the study. After these exclusions, 31 patients with CSU were finally enrolled in the study.

The control group included 22 healthy children who were periodically attending pediatric welfare clinics at the same hospitals for regular checkups. Children in the control group did not have a history of chronic or acute urticaria, had never been diagnosed with any allergic disease, and were not taking any medications. In addition, no signs of infectious disease were present in any of the subjects in the control group.

Assessment of Urticarial Activity

Disease activity was evaluated using the urticaria activity score (UAS) according to the EAACI guidelines [14]. These scores were calculated in interviews with patients and families, as well as during the physical examination. The UAS consisted of the sum of the wheal number score and the pruritus score, which ranged from 0 to 6.

Blood Sample Collection

Patients were instructed not to consume foods with high nitrite contents for 2 days before blood sampling, such as cured meat products, canned vegetables, and some fresh vegetables that contain more than 100 mg of nitrate/100 g fresh weight, including celery, beetroot, spinach, lettuce, carrots, rocket, parsley, radishes, and leeks [15, 16]. Antihistamines and montelukast were discontinued for at least 24 h before blood sampling. After overnight fasting, peripheral blood samples (4 ml in total) were collected from an antecubital vein into heparinized tubes; thereafter, blood was centrifuged at 3,000 g for 10 min to obtain the plasma. The separated plasma was frozen immediately at -80°C until further analysis of plasma total NOx metabolites, nitrite, and nitrate levels.

Laboratory Investigations

The following parameters and tests were considered as routine laboratory procedures: erythrocyte sedimentation rate, complete blood count, liver function tests, urea, creatinine, electrolytes, thyroid-stimulating hormone, free thyroxine, antithyroid peroxidase and antithyroglobulin antibodies, total immunoglobulin E, urinalysis with test strips and urine cultures, microscopic investigation of stool for parasites, stool enzyme immunoassay for *Helicobacter pylori* antigen, and skin prick tests for aeroallergens and food allergens. In addition, the autologous serum skin test (ASST) was performed on the patients using the method described by Sabroe et al. [17].

Measurement of Plasma NOx, Nitrite, and Nitrate Levels

Plasma levels of NOx, nitrite, and nitrate were determined via the nitrate reductase enzyme using a Griess method-based commercial kit (NO colorimetric assay, Roche Diagnostics GmbH, Mannheim, Germany). Samples were thawed at room temperature, then equal volumes of serum and potassium phosphate buffer were placed in an ultrafilter and centrifuged at 4,000 rpm for 45 min. The ultrafiltrate was collected and used in the test. Nitrates were quantitatively converted to nitrites for analysis. Enzymatic reduction of nitrate to nitrite was carried out using coenzymes (NADPH, FAD) in the presence of nitrate reductase in the incubation assay step. N-1-(naphthyl)ethylenediamine dihydrochloride, sulfanilamide, and incubation solutions were mixed at a ratio of 1:1:2 (v/v). These mixtures were incubated for 5 min at room temperature in dimmed light and measured at 550 nm using a Varioskan™ Flash Multimode Reader (ThermoScientific™, Waltham, MA, USA). Sodium nitrite of 1.00 mM was used as the standard for the determination of nitrite, and potassium nitrate of 80 mM was used as the standard for the determination of nitrate.

Table 1. Some demographic and clinical features of the study group

	CSU patients (n = 31)	Control group (n = 22)	p value
Mean age $\pm$ SD, years	11.3 $\pm$ 3.9	10.2 $\pm$ 2.9	ns
Gender (M/F), n	16/15	10/12	ns
Aeroallergen sensitization, n (%)	4/31 (13)	nd	
Total IgE >100 kU/L, n (%)	12/31 (38)	nd	
Eosinophils >5%, n (%)	3/31 (10)	nd	
ASST positivity, n (%)		nd	
ASST positive	9/31 (29)		
ASST negative	22/31 (71)		
Median UAS (IQR)	4 (2–5)	–	
Treatment, n (%)		–	
Second-generation antihistamines at licensed doses	20 (64)		
Second-generation antihistamines at high doses	3 (10)		
Combination of second-generation antihistamines and montelukast	8 (26)		

CSU, chronic spontaneous urticaria; SD, standard deviation; IQR, interquartile range; ASST, autologous serum skin test; UAS, urticaria activity score; nd, not done; ns, not significant.

Ethical Approval

The study was performed in accordance with the tenets of the Declaration of Helsinki and good clinical practice, and it was approved by the Bezmialem Vakif University Ethical Committee (No. 16234). All study participants and their parents were given information about the study, and signed consent was obtained from the parents.

Statistical Analyses

A statistical analysis was performed using IBM SPSS version 22 (IBM, Armonk, NY, USA). A Shapiro-Wilk test was used to test distributions for normality. Parametric data were expressed as the mean $\pm$ standard deviation, and nonparametric data were expressed as the median and interquartile range (IQR). Comparisons between group means was carried out using the 1-way analysis of variance (ANOVA) and Mann-Whitney U tests. The correlation between 2 variables was assessed using the Spearman rank correlation coefficient. Categorical data were evaluated using the χ^2 test. $p < 0.05$ was accepted as being statistically significant.

Results

General Characteristics

The study group consisted of 16 boys and 15 girls, while the control group comprised 10 boys and 12 girls. The mean ages of the patients with CSU and the control group participants were 11.3 ± 3.9 and 10.2 ± 2.9 years, respectively. No significant differences in age or gender was found between the groups ($p > 0.05$). Biochemical analysis, erythrocyte sedimentation rate, thyroid-stimu-

lating hormone, free thyroxine, antithyroid peroxidase and antithyroglobulin antibodies, and urinalysis of all the CSU patients were within normal limits. Food prick tests, urine cultures, stool tests for parasites, and *H. pylori* results were negative in all patients. The median value of the UAS was 4 (IQR 2–5), and 29% of the patients were ASST positive. Some demographic and clinical features of the patients and the control group are shown in Table 1.

Plasma NOx, Nitrite, and Nitrate Levels

The values of the plasma NOx levels were 82.5 ± 11.3 $\mu\text{mol/L}$ in the CSU group and 50.9 ± 9.4 $\mu\text{mol/L}$ in the control group. There was a statistically significant difference between the groups ($p < 0.001$; Fig. 1). The CSU group also had statistically significantly elevated plasma nitrite levels (53.3 ± 13.8 vs. 30.2 ± 10.1 $\mu\text{mol/L}$, respectively, $p < 0.001$; Fig. 1). The median values of plasma nitrate were 27.5 $\mu\text{mol/L}$ (IQR 19.1–35.5) in CSU patients and 20.9 $\mu\text{mol/L}$ (IQR 17.9–23.2) in the control group. The difference between the groups was statistically significant ($p = 0.009$; Fig. 1). Neither the UAS nor plasma NOx, nitrite, or nitrate levels were significantly different between the ASST-positive and ASST-negative patients ($p > 0.05$). In addition, plasma NOx and nitrite levels showed a statistically significant positive correlation with the UAS ($\rho = 0.512$, $p = 0.03$ and $\rho = 0.452$, $p = 0.011$, respectively; Fig. 2). However, there was no significant correlation between plasma nitrate levels and the UAS ($p > 0.05$).

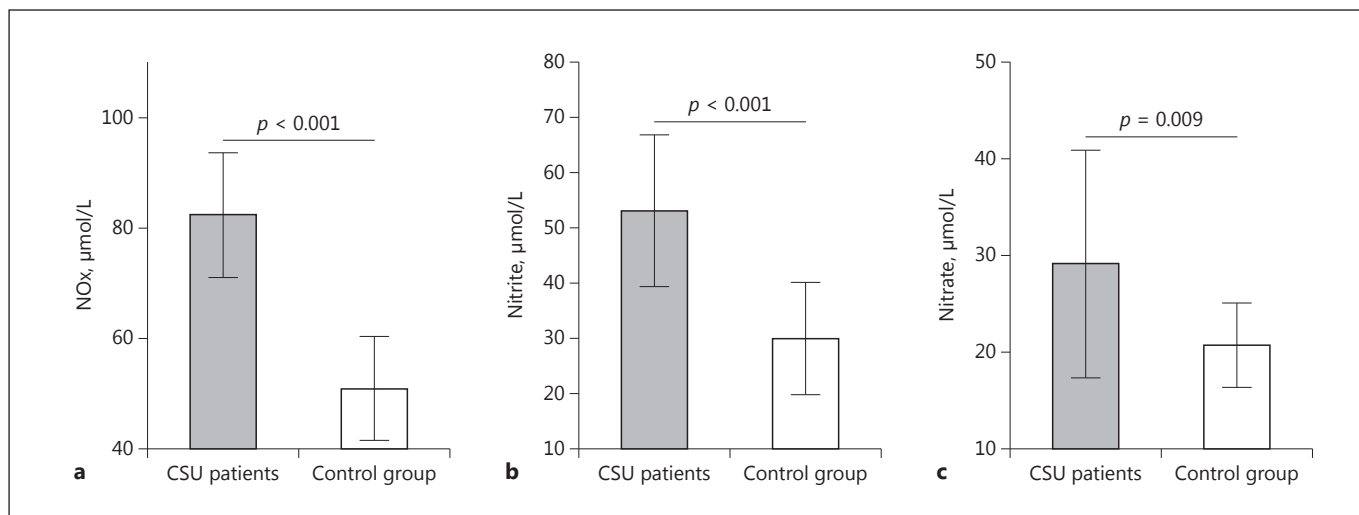


Fig. 1. Plasma levels of total nitric oxide metabolites (NOx; **a**), nitrite (**b**), and nitrate (**c**) in CSU patients and healthy controls.

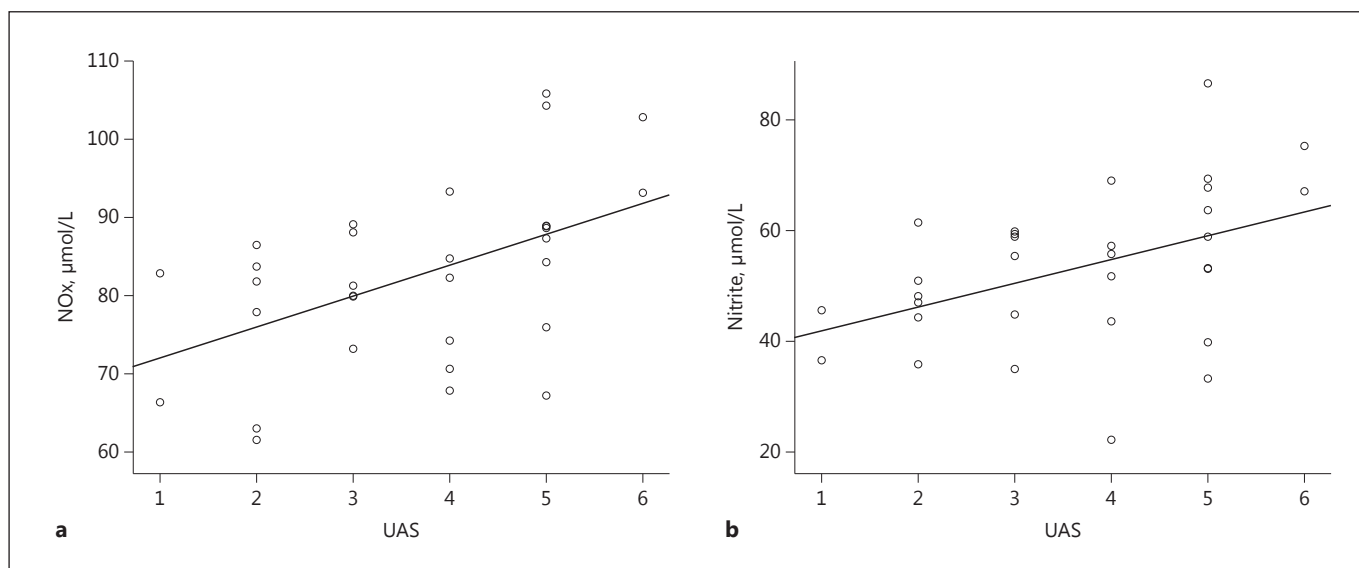


Fig. 2. Positive correlation between the urticaria activity score and plasma levels of total nitric oxide metabolites (NOx; **a**) and nitrite (**b**).

Discussion

Our study results showed that patients with CSU had elevated NS levels compared to controls and that there was a positive correlation between plasma levels of NOx and nitrite with disease activity. Another distinctive feature of our research is that it was conducted in children, and the prevalence of CU in children is much lower than that in adults [1, 18, 19]. Therefore, only a few studies re-

lated to the pathogenesis of CU have been performed in children, and ours is the first study on this subject.

The activity of CSU can be assessed both by the physician during the examination (UAS) and by patients or families by documenting the UAS for 7 days consecutively (UAS7) [2, 14]. UAS7 represents full family or patient-derived information and provides us with an overview of the overall disease activity. We employed the UAS in this study because we aimed to compare momentary activity

scores and plasma NOx levels [20]. In addition, we aimed to reduce or eliminate subjective assessments provided by families in the scoring as much as possible.

No objective biomarker of disease activity or response to treatment in CSU patients has been previously identified. In our study, plasma NOx and nitrite levels showed statistically significant positive correlations with the UAS. Conversely, we did not detect the same correlation between nitrate levels and the UAS ($p > 0.05$). In particular, NOx levels showed a more powerful correlation with the UAS than did nitrite levels. Although further studies are needed, plasma levels of NOx may be an ideal biomarker for evaluating disease severity in CSU patients.

Although the Griess assay is a simple, reliable, and widely used method, it undoubtedly has some limitations. Liver and kidney diseases and nitrates taken with food can affect the plasma nitrate levels [16, 20]. In this study, all patients were evaluated for possible etiological factors of CU; thus, patients' renal and hepatic functions were evaluated and found to be normal. In addition, patients and controls were instructed not to consume the high nitrate-containing foods in our country's cuisine for 2 days before blood sampling, which is the suggested period in similar studies in the literature [16].

NO is a free radical molecule that includes an unpaired electron; it is a reactive nitrogen species, along with other members of this group, such as nitrogen dioxide and peroxynitrite [21]. NO is synthesized from L-arginine via NOS, which exist in 3 isoforms [6]. The neuronal (NOS 1) and endothelial (NOS 3) isoforms of the enzyme are expressed constitutively and inhibited by NO synthesized by itself; therefore, they cause only low-level NO production [21]. However, the activity of isoform 2 (iNOS) is regulated via gene expression and requires stimulation before its expression. iNOS is expressed in all inflammatory cells and can produce NO in excessive amounts (about 1,000 times that of the other isoforms) [21, 22]. NO has striking antimicrobial and pro- and anti-inflammatory roles in both the innate and adaptive immune systems [5, 6, 23]. Although it is not completely understood, it seems low amounts of NO exert beneficial anti-inflammatory effects, but large quantities of NO are proinflammatory and can cause elevated vascular permeability, leukocyte infiltration, and even tissue damage [5, 23].

Wheals, flares, and angioedema, which are typical clinical presentations of CSU, develop as a result of increased vasopermeability [24], and this phenomenon is mainly ascribed to histamines. However, almost half of CSU patients do not respond sufficiently to licensed doses of an-

tihistamines [18]. Moreover, it is known that some inflammatory mediators, such as complement component 5a and leukotriene B₄, may also cause increased vasopermeability [1]. It is most likely that other – even undiscovered – mediators, such as reactive oxygen and nitrogen species, play critical roles in this phenomenon [25].

NO is a potent vasodilator for dermal microcirculation [26] that can also increase vascular permeability [27, 28]. Previous reports have revealed widespread expression of NOS enzymes in inflammatory and endothelial cells in inflammatory dermatoses, such as atopic dermatitis, allergic contact dermatitis, pemphigus, and psoriasis [29–31]. In a previous report, the authors described the elevated expression of iNOS in keratinocytes during the course of acute urticaria [32]. Only 1 study conducted on adults reported elevated serum NOx levels in patients with acute urticaria compared to healthy subjects [33].

Studies have shown that NO can be synthesized in mast cells and that it has regulatory roles in leukotriene synthesis and phagocytosis [34, 35]. However, whether the mast cells can release NO to the extracellular space is still a controversial issue [21, 34, 36, 37]. In addition, lesional skin biopsies obtained from CSU patients revealed the infiltration of perivascular CD4⁺ lymphocytes, monocytes, and granulocytes [1]. Assessing the literature data and our results together, it is plausible to suggest that the elevated plasma NO levels in CSU originate from the mentioned cells or vascular endothelial cells instead of mast cells. NO has double-sided effects in allergic inflammation. On the one hand, it inhibits chemokine production in mast cells [21, 38], while on the other hand it induces vasodilatation, vasopermeation, and leukocyte-endothelial adherence [38]. We think that the proinflammatory properties of NO outweigh its anti-inflammatory properties in CSU, as in the case of asthma [39].

Inhibition of iNOS has resulted in potent anti-inflammatory effects in almost all animal models tested [40]. In an asthma model, it has been shown that iNOS inhibitors impede inflammatory cell infiltration and microvascular leakage [41]. Therefore, the therapeutic efficacy of NOS inhibition is a highly active area of research in the treatment of various diseases, such as septic shock, migraine, Alzheimer disease, asthma, and chronic obstructive pulmonary disease [40, 42–45]. Hundreds of NOS inhibitor drugs have been patented in the past few years [45]. If larger clinical studies also prove the role of NS in its pathogenesis, CSU may be another research area for investigating the therapeutic values of NOS inhibitors in the near future.

In conclusion, children with CSU have elevated plasma NOx, nitrite, and nitrate levels compared to healthy controls. In addition, plasma levels of NOx and nitrite are positively correlated with disease activity. NS may be one of the unrevealed parts of CSU pathogenesis; it may also represent a possible therapeutic target. To our knowledge, this is the first study on this issue, and many more studies are needed before a final conclusion can be made on this topic.

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Disclosure Statement

The authors report no conflicts of interest in relation to this paper.

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