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Can tissue nitric oxide synthesis 2 (iNOS) levels play a role in the pathophysiology of reflux esophagitis?

Enver Akbaş[1]*, Gözde Ülfer[2]

1 Dept. of Gastroenterology, Medipol University Faculty of Medicine, Istanbul, TR
2 Dept. of Biochemistry, Medipol University Faculty of Medicine, Istanbul, TR

*** Corresponding Author:** Enver Akbaş **E-mail:** drenverakbas@gmail.com

ABSTRACT

Objective: Nitric oxide (NO) is a strong dilatator, playing an important role in inflammatory events. Its production is regulated by NO synthase 2 (NOS2/iNOS). Our aim was to compare iNOS in esophageal tissues of patients with erosive or non-erosive reflux esophagitis to that of normal cases.

Materials and Methods: The study was conducted in 2019–2020 on patients undergoing upper gastrointestinal (UGI) endoscopy. Study included 30 patients who had no reflux symptoms and were not diagnosed with reflux esophagitis in the UGI endoscopy (control), 22 who had pronounced reflux symptoms but could not be diagnosed with reflux esophagitis in the endoscopy (non-erosive reflux), and 51 who had reflux esophagitis in the endoscopy (erosive reflux esophagitis). Using the enzyme-linked immunosorbent assay, tissue iNOS levels were assessed on samples from the lower end of the esophagus.

Results: Average iNOS level was 5.02±1.51 picogram/milliliter (pg/mL) in the normal group and 5.04±1.68 pg/mL in all reflux esophagitis cases. iNOS levels were higher in non-erosive reflux and lower in erosive reflux than in controls. In erosive reflux A, B, and C, iNOS levels were 5.03±1.64, 5.10±2.23, and 4.06±0.02 pg/mL, respectively. The level in erosive reflux C is considerably lower than in the normal group. However, none of the differences between the groups was significant.

Conclusions: NO synthase was higher in patients with non-erosive reflux esophagitis and considerably lower in those with erosive reflux C, compared to the normal cases. Although not significant, the differences suggest that NO and iNOS levels may be important in reflux physiopathology.

Keywords: Nitric oxide synthase, Nitric oxide, Gastroesophageal reflux

INTRODUCTION

Among the most common disorders in the gastrointestinal tract is gastroesophageal reflux disease (GERD) (1, 2). Research has revealed that about 20% of adults in American culture have reflux symptoms, such as burning in the chest and acid regurgitation, at least once every week (3, 4). Gastroesophageal reflux disease occurs if the content in the stomach gets back to the esophagus, causing the person to experience a number of disturbing symptoms and/or health issues (5). Reflux esophagitis, in other words, is a reflux condition that develops in cases where the damage done by acid, pepsin, and bile cannot be mitigated by the mechanisms of the mucosal defense system. The mucosa may be usual or mildly erythematic in non-erosive reflux disease (NERD). There is evident mucosal damage in the case of erosive esophagitis. The damage is characterized with redness, friability, superficial linear ulcers, and exudation (6). Reflux esophagitis is, in other words, the consequence of an inflammatory process in the mucosa of the esophagus.

Smooth muscles and vascular structures may be affected by the strong dilator characteristic of nitric oxide (NO). While its mechanism is not yet clearly known, it may have a role to play in the development of reflux esophagitis. Moreover, NO is a soluble endogenous gas. It has a variety of biological functions, including signaling. It functions as an effector molecule or metabolic regulator. Immune myeloid cells like macrophages

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stimulate the supply of cytokines and NO in response to inflammation, causing signals that are important for the eradication of pathogens (7).Nitric oxide synthases (NOSs) are the isoform family responsible for NO synthesis. Inducible NOS (iNOS) expression takes place in inflammatory conditions, and under such conditions, NO is generated in high amounts. INOS is viewed to be a harmful enzyme in pathological conditions and is believed to be one of the major parameters affecting the development of cardiovascular system diseases like atherosclerosis (8). Nitric oxide generated by the inducible isoform of nitric oxide synthase (iNOS) has very complicated role. The induction of iNOS expression and, therefore, NO creation has been identified to have desirable affects that destroy viruses, parasites, microbes, and tumors and that are immunomodulatory. But iNOS leads to harmful consequences and seems to play a role in the pathophysiology of different human diseases if induced in the wrong spot and at the wrong time (9). The role of NO and iNOS in the inflammatory process of esophageal mucosa in GERD is not a subject that has been sufficiently investigated. In our study, we explored whether iNOS production in patients with reflux esophagitis differs from that in normal people due to the dilatator effect of NO and its responsibility in inflammatory processes. Thus and so, we assessed the role of iNOS levels of esophageal tissues in the pathophysiology of reflux esophagitis and attempted to identify its potential to become a parameter for diagnosis if it had a role to play.

MATERIAL and METHODS

The study was carried out between 2019 and 2020 on 103 patients who presented to our clinic and underwent upper gastrointestinal (UGI) endoscopy for various of reasons. Prior to the study, approval was obtained from the ethics committee of our institution. Ethical approval for this study (Ethical Committee decision No;198) was provided by the Ethical Committee of İstanbul Medipol University Hospitals, on 22 March 2019. The study was designed and carried out as a prospective study. Patients were included in the study voluntarily, and each patient was required to sign an informed consent form. The study included 30 patients who had no reflux symptoms and were not diagnosed with reflux esophagitis in the UGI endoscopy as the control group, 22 who had pronounced reflux symptoms but could not be diagnosed with reflux esophagitis in the endoscopy as the non-erosive reflux group, and 51 who had considerable reflux esophagitis in the endoscopy as the erosive reflux esophagitis group.

The Los Angeles (LA) classification was used to screen the patients endoscopically and stage reflux esophagitis. Patients in the non-erosive reflux group were included in the study after 24-hour pH-metry confirmed the diagnosis of reflux. Samples of tissue were taken from all patients in the case and control groups using biopsy forceps from the lower end of the esophagus about 5 cm above the Z-line. The samples were put in a tube without subjecting to any solution or processing and kept at –80 degrees until the study was completed. At the final phase of the study, iNOS levels of the tissue samples were studied using commercial kits produced by Cloud-Clone Corp. and the enzyme-linked immunosorbent assay (ELISA) method.

Statistical Analysis

IBM SPSS 22 statistics program was used for statistical analysis to assess the findings obtained in the study. Descriptive statistical methods (means, standard deviations, medians, frequencies, ratios, and minimum and maximum values) were used when assessing the data of the study. Whether the quantitative data were normally distributed was tested using Kolmogorov-Smirnov test, Shapiro-Wilk test and graph analyses. The student t test was used for pairwise comparisons of quantitative data with normal distribution, and the Mann Whitney U test was used for pairwise comparisons of data not normally distributed. One-Way ANOVA test was used in comparisons of three or more groups with normal distribution, and Bonferroni test was used in their pairwise comparisons. Kruskal Wallis test was used in comparisons of three or more groups that were not normally distributed, and Bonferroni-Dunn test was used in their pairwise comparisons. Pearson's Chi-Square test was used to compare qualitative data. Significance was assessed at least at the $p < .05$ level.

RESULTS

This study was conducted on a total of 103 cases, 37.9% (n = 39) female and 62.1% (n = 64) male. The ages of the cases ranged from 19 to 61 years, and the mean age was 37.50 ± 10.72 years. **Table 1** illustrates the percentage-wise distribution of cases by group.

The demographic data of the control group and the reflux esophagitis group by gender and age are shown in **Table 2.**

With regard to age, there was no statistically significant difference between the normal cases in the control group and the cases in all groups of reflux esophagitis ($p > .05$). No statistically significant age difference was found between the patients in the normal group and the non-erosive and erosive reflux groups either ($p > .05$). A statistically significant difference was found between the gender distributions of the normal cases and the patients with reflux esophagitis ($p = .003$; $p < .01$). Regardless of gender, the rate of male reflux esophagitis patients is higher among randomly recruited cases.

The average iNOS level of esophageal tissues in all cases is 5.04 ± 1.62 picogram/milliliter (pg/mL). The average was 5.02 ± 1.51 pg/mL in the normal group, while it was 5.04 ± 1.68 pg/mL in the group with all reflux esophagitis cases. There was no statistically significant difference between tissue iNOS measurements of normal and all reflux esophageal group patients ($p > .05$).

A separate evaluation of the normal, non-erosive reflux and erosive reflux groups revealed that the average iNOS level was 5.02 ± 1.51 pg/mL in the normal group, 5.39 ± 1.62 pg/mL in the non-erosive reflux group, which was high, and 4.89 ± 1.70 pg/mL in the erosive reflux group, which was low. However, the differences between the tissue iNOS levels of these cases are not statistically significant ($p > .05$).

Table 3 shows the presentation of data from sub-types of reflux esophagitis and control group cases.

When the cases in the groups were examined according to the type of reflux, the iNOS level was 5.02 ± 1.51pg/mL in the

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normal group and 5.39 ± 1.62 pg/mL in the non-erosive group, the latter of which was considerably higher. Next, the cases in the erosive reflux group were evaluated among themselves. While the iNOS level was 5.02 ± 1.51 pg/mL in the normal group, it was 5.03 ± 1.64 pg/mL in erosive reflux A, and 5.10 ± 2.23 pg/mL in erosive reflux B, and 4.06 ± 0.02 pg/mL in erosive reflux at C. The iNOS level was considerably lower in erosive reflux C. However, the differences between the iNOS measures of these different groups are not statistically significant either ($p > .05$). **Figure 1** shows the graphical representation of the iNOS levels in tissues of normal cases and cases in non-erosive reflux and erosive reflux subgroups.

At the beginning of the study, it was planned that patients with LA Stage D and those with Barret esophagitis who had complications would also be included in the erosive reflux esophagitis group in this study. However, no patients of this group could be included in the study because they did not present to our center during the period the study was conducted.

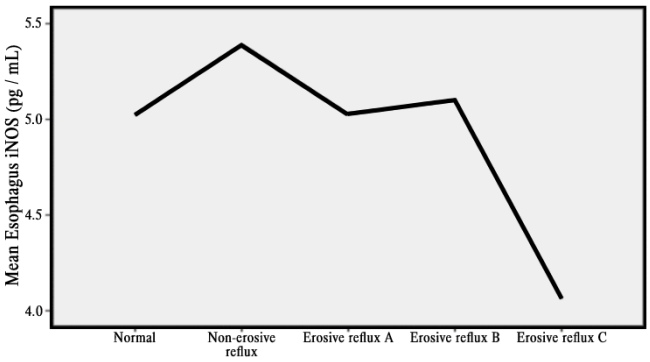

Figure 1. INOS levels in tissues of normal, non-erosive reflux and erosive reflux group cases

Table 1. Case distribution by group

Groups	Total (n = 103) n	%
Control Group	30	29.1
Non-Erosive Reflux Group	22	21.4
Erosive Reflux LA Stage A	27	26.2
Erosive Reflux LA Stage B	15	14.6
Erosive Reflux LA Stage C	9	8.7

Table 2. Demographic data of cases in the control and reflux esophagitis groups

		Reflux esophagitis (n = 73)	
	Normal (n = 30)	Non-Erosive Reflux (n = 22)	Erosive Reflux (n = 51)
Age (Years)			
Min–Max (Median)	19–51 (33.00)	19–56 (36.50)	24–61 (38.00)
Mean ± SD	33.00 ± 10.51	37.32 ± 9.88	39.15 ± 10.63
Gender, *n (%)*			
Female	18 (60.00)	12 (54.50)	9 (17.60)
Male	12 (40.00)	10 (45.50)	42 (82.40)

Table 3. Analysis of data according to reflux esophagitis sub-groups

		Reflux esophagitis (n = 73)				
	Normal (n = 30)	Non-Erosive Reflux (n = 22)	Erosive Reflux A (n = 27)	Erosive Reflux B (n = 15)	Erosive Reflux C (n = 9)	*p*
Age (Years)						
Min–Max (Median)	19–51 (33)	19–56 (36.5)	26–61 (37)	24–55 (33)	30–52 (50.5)	[a]***0.214***
Mean ± SD	33.00 ± 10.51	37.32 ± 9.88	38.33 ± 10.58	37.31 ± 10.99	44.88 ± 9.46	
Gender, *n (%)*						
Female	18 (60.0)	12 (54.5)	4 (14.8)	2 (13.3)	3 (33.3)	[b]***0.001****
Male	12 (40.0)	10 (45.5)	23 (85.2)	13 (86.7)	6 (66.7)	
Esophageal iNOS (pg/mL)						
Min–Max (Median)	3–7.9 (4.9)	2.7–10 (5.3)	3.1–9.2 (4.5)	1.8–9.1 (5.9)	4.1–4.1 (4.1)	[a]***0.197***
Mean ± SD	5.02 ± 1.51	5.39 ± 1.62	5.03 ± 1.64	5.10 ± 2.23	4.06 ± 0.02	

*[a]Kruskal Wallis Test; bPearson Chi-square Test *p < .01*

DISCUSSION

Nitric oxide is an important mediator of processes that are physiological and pathological. NO has direct and indirect effects. Direct effects occur between NO and its specific biological molecules, while indirect effects are mediated by the reactive types of nitrogen oxide (RNOS) consisting of NO reactions with oxygen or superoxide (**10**). NO is synthesized from L-arginine in the living organism. It is a soluble enzyme, and its production is catalyzed by iNOS and active in its dimeric form. It is an important biological mediator. However, it is cytotoxic if produced too much. Cytokines stimulating the immune system and bacterial pathogens activate iNOS and generate high amounts of NO by activating inducible nuclear factors (**11**). In macrophages, NO is generated by iNOS as an outcome of stimulation by microbes and cytokines. NO is required for the protection of the host and immune regulation against pathogens (**12**). INOS can be most easily detected in monocytes or macrophages of people suffering from inflammation or infections. Continuous NO generation provides macrophages with activity that inhibits cell growth or is toxic to living cells, against viruses, bacteria, fungi, protozoans, parasitic worms, and tumor cells. The high-output NO pathway is likely to have evolved to defend the host from being infected; nevertheless, it grants iNOS the dilemma of both protective and destructive immune response through its ability to suppress lymphocyte multiplication and to harm other normal cells of the host (**13**). For this reason, the production of NO through iNOS stimulation appears to lead to different and contradictory consequences for different organ and tissue systems. In Barret esophagus and esophageal adenocarcinoma, which develops on the basis of gastroesophageal reflux, inflammatory disorders are thought to have the potential cause carcinogenesis through activation of genes that are prosurvival, including cyclooxygenase-2 (COX-2) and iNOS. Yet, Heather et al. determined no significant relationship between iNOS polymorphism and Barret esophagus or reflux esophagitis (**14**). NO, generated by iNOS, has had a role to play not only in the induction of DNA damage but also in abnormal signaling of cells in a variety of previous tissue and cell studies. Inflammatory mediators like NO may have a major role in the development of esophageal cancer because esophageal adenocarcinoma is caused by gastroesophageal reflux disease in the context of Barrett's esophagus. McAdam et al. have confirmed in their study that iNOS protein levels are increased in esophageal adenocarcinoma emerges on the background of reflux esophagitis and therefore in the development of neoplasms in the esophagus (**15**). NO level in the intestinal tract increases during inflammation, possibly contributing to intestinal injuries. It is unclear, however, how the expression of two forms of messenger ribonucleic acid (mRNA) — iNOS and endothelial NO synthase mRNA — in the esophageal mucosa adds to the damage to the mucosa that reflux esophagitis causes. Inamori et al. has found that iNOS mRNA expression in the mucosa of the esophagus is intensified as a function of the gravity of esophagitis and argued that the buildup of NO induction by iNOS has something to do with reflux esophagitis exacerbation (**16**).

In our study, the patients with non-erosive reflux esophagitis were found to have higher levels of iNOS in the esophageal tissue than those in the control group but lower in comparison to those with erosive reflux C. In addition, these iNOS values did not differ statistically significantly. However, if the cases in the erosive reflux D and Barret esophagus groups could be included in the study, the results could have changed, which is a limitation of our study. Moreover, there are no specifically defined thresholds for iNOS and NO amounts in normal esophageal tissues. In our study, the iNOS levels in esophageal tissues were about 5 pg/mL, which is probably within sensitive limits. If we take the average level of 5.39 pg/mL observed in patients with non-erosive reflux in our study to be elevated, the potential high NO levels due to high iNOS levels could cause patients to have more reflux symptoms due to the dilator effect of NO on the smooth esophageal muscles. In patients with erosive reflux C, however, the iNOS levels were found to be 4.06 pg/mL, which was lower than normal. Minimal increase in expression of NOS may increase extensive production of NO. So, minimal changes in NOS may effect different metabolic processes (**17**). These results suggest the necessity of further research into the role of lower esophageal sphincter relaxation, which may occur via the NO pathway in the pathophysiology of reflux esophagitis as well as other mechanisms.

CONCLUSIONS

Low iNOS and NO levels may be impairing the blood supply to the microvascular bed in the mucosa of esophagus in these patients, contributing even more to the gastric acid-induced damage to the esophagus. Moreover, a low microvascular supply of blood to esophageal tissues may be leading to a decrease in their acid clearance. Comprehensive studies involving a more detailed and broader group of cases are needed to prove all these theories and to reveal the role of iNOS in reflux esophageal pathophysiology.

Human Rights Statement and Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and later versions. Informed consent or substitute for it was obtained from all patients for being included in the study.

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