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Influence of Topical N-Acetylcysteine Therapy on Macrophage Polarization Markers in Chronic Rhinosinusitis Patients

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Abstract

Chronic rhinosinusitis (CRS), a prevalent inflammatory illness, affects a sizeable portion of the global population. An important component of the upper respiratory system, nitric oxide (NO) controls inflammatory responses and functions as an antibacterial, antiviral, and antifungal agent. The enzymes arginase (ARG) and inducible nitric oxide synthase (iNOS), whose activity has been linked to a range of respiratory disorders, generate NO. N-acetylcysteine has demonstrated potential as a CRS treatment option. The present study aimed to evaluate the levels of iNOS, ARG, and constitutive isoforms of NO-synthase in sinonasal fluid at multiple time points after surgery. Significant differences were observed in the levels of iNOS, ARG, and constitutive isoforms of NO-synthase between the two groups after treatment. The experimental group, receiving topical N-acetylcysteine, exhibited increased iNOS activity on the 3rd day, followed by a decline on days 10 and 28. Similarly, ARG activity increased on day 3rd in both groups but was lower in the experimental group at later time points. The findings suggest that NO-related enzymes, particularly iNOS and ARG, play a crucial role in the pathophysiology of chronic rhinosinusitis without polyps. Additionally, topical N-acetylcysteine treatment seems to affect NO production and ARG activity, which may help to reduce inflammation and enhance clinical results. These results highlight the therapeutic potential of N-acetylcysteine in managing chronic rhinosinusitis and warrant further investigations into its broader implications for respiratory conditions. For patients with CRS without polyps, adding N-acetylcysteine to the treatment regimen may result in more focused and efficient therapeutic strategies.

Keywords: Nasal cavity, Nasal obstruction, Nasal division, Mucociliary transport, Clinical presentation, Maxillary sinus

Introduction

Research Problem

One of the most common chronic conditions is chronic rhinosinusitis, which affects between 5% and 12% of the general population (de Loos *et al*., 2019). It is an inflammatory disorder characterized by paranasal sinus inflammation and mucosal lining mucosa lasting at least 12 weeks (Rosenfeld *et al*., 2015; Papadakis *et al*., 2021). Expectedly, such a chronic condition necessitates regular trips to an otorhinolaryngology clinic and significantly affects health burden and quality of life globally.

Research Focus

Nitric oxide (NO), which is believed to play a protective role for the upper respiratory system by acting as an antibacterial, antiviral, and antifungal agent as well as by increasing mucociliary clearance and regulating inflammatory response, is a primary source in the paranasal sinuses (Taruya *et al*., 2015) and lower airways (Ricciardolo, 2003). Low nasal NO levels have reportedly been observed in chronic (Taruya *et al.*, 2015) or acute rhinosinusitis (Lanz *et al.*, 2008) and primary ciliary dyskinesia (Wodehouse *et al*., 2003), but high in allergic rhinitis (Lee *et al.*, 2012). Inducible nitric oxide synthase (iNOS) catalyzes the L-arginine oxidation process, which leads to the endogenous production of NO. Arginase (ARG) uses the amino acid L-arginine in the urea cycle to produce urea and ornithine (Vlad *et al*., 2015), As a result, the two enzymes are vying for the same substrate. There are two arginine isoforms: arginine 1 (ARG1), and arginine 2 (ARG2), respectively. In the pathophysiology of several airway diseases, it appears that the L-arginine-nitric oxide pathway modulates several mechanisms that regulate the expression of the ARG1 and ARG2 genes (Luiking *et al*., 2012).

The 2012 European Position Paper on Rhinosinusitis (EPOS), which recognizes three sorts of sinusitis, is presently used to order persistent rhinosinusitis. Persistent rhinosinusitis with nasal polyps (CRSwNP), unfavorably susceptible parasitic sinusitis (AFS), and constant rhinosinusitis without nasal polyps (CRSsNP) (Fokkens *et al*., 2012). In response to the global pandemic of COVID-19, which is associated with symptoms related to inflammation and venous congestion, both iNOS expression and NO production may rise (Cinar *et al*., 2022). However, more research has shown that distinct COVID-19 illness waves have varied effects on iNOS activity, and in certain situations, iNOS activity and nitric oxide synthesis are even deficient (Gelzo *et al*., 2022). Patients with recurrent COVID-19 or those who have already had COVID-19 but do not exhibit symptoms have lower iNOS activity, which is correlated with a drop in tetrahydrobiopterin concentration and

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the dissociation of iNOS from its substrate (Villaume, 2022). Long-term immunosuppression follows a chronic COVID-19 infection, which may be followed by the fibrosis of multiple organs and the emergence of opportunistic infections such as rhino-orbital-cerebral mucormycosis (Bhattacharyya *et al*., 2021; Oronsky *et al*., 2023). Additionally, profibrotic macrophages (polarised as M2 phenotype) predominate in peripheral tissues (Islam *et al*., 2023). Nasal block, facial pressure or pain, postnasal drip or nasal discharge, anosmia or hyposmia, and other clinical signs of chronic rhinosinusitis like Erythema or edema, discharge, and nasal polyps or polypoidal mucosa, are endoscopic signs. The Lund-Mackay bilateral scoring system is used to score computed tomography (CT) images for radiological signs. Contingent upon the seriousness of the clinical infection and the patient's response, there are a few treatment decisions, including restorative, careful, or a blend of both (Uvaliyeva, 2022).

N-acetylcysteine is a well-known medication used for treating conditions because of its mucolytic effects, but it also possesses strong antioxidant properties. It improves detoxification, increases glutathione production, and directly scavenges free radicals. Other effects have also been noted, including the restoration of immune cell response, control of the inflammatory response, prevention of thrombosis, and exertion of an antiviral impact (Calzetta *et al*., 2018; Meirmanova, 2022). N-acetylcysteine has also been linked to advantages for respiratory outcomes (Sharafkhah *et al*., 2018) and other end-organ complications (Khan *et al*., 2021). N-acetylcysteine can decrease transforming growth factor- signaling because of N-acetylcysteine. This reduces the development of fibrosis and enables tissue macrophages to adopt a distinct (different from profibrotic M2-polarization) morphology (Yazaki *et al*., 2021). However, this only applies to macrophages whose M1 polarisation has been induced by bacterial lipopolysaccharides, i.e. pathogen-associated molecular patterns (PAMPs), as N-acetylcysteine generally promotes the polarisation of macrophages towards the M2 phenotype and prevents their polarization towards the M1 (pro-inflammatory) phenotype (Ren *et al*., 2020). After strong acids and other substances have caused cell damage, N-acetylcysteine can reduce the generation of further damage-associated molecular patterns (DAMPs) (Fujiwara *et al*., 2021). N-acetylcysteine is a reasonable therapeutic alternative for COVID-19 due to the limitations and occasionally inadequacies of present medications. It also has potential implications for disease development. As a result, early research suggests that N-acetylcysteine benefits clinical outcomes in COVID-19 patients and may be used as a component of multimodal care for these patients (Ibrahim *et al*., 2020; Avdeev *et al*., 2022).

Research Aim and Research Questions

In light of the above-mentioned facts, the purpose of the current study was to determine the concentration of the induced form of NO-synthase, arginase, and constitutive isoforms of NO-synthase in the nasal mucosa of patients with chronic rhinosinusitis who did not have polyps.

1. What are the effects of N-acetylcysteine on NO synthesis and ARG activity in CRS patients without polyps?
2. Can topical N-acetylcysteine administration lead to reduced inflammation and improved clinical outcomes in individuals with chronic rhinosinusitis without polyps?
3. How do the levels of iNOS, ARG, and constitutive isoforms of NO-synthase change over time after surgical therapy in CRS patients without polyps?

Materials and Methods

Study Design

A randomized controlled study design was used for a population of 96 patients aged 18 to 60 years with a diagnosis of chronic rhinosinusitis after surgical treatment. From the selected population, 47 patients who received irrigation of the topical form of N-acetylcysteine and 49 patients who received topical therapy using irrigation of 0.9% NaCl solution were randomly divided into two groups. The current study was conducted at Bogomolets National Medical University's Department of Otorhinolaryngology in collaboration with the CNME "Kremenchuk City Hospital of Planned Treatment, Kremenchuk" and the Oleksandriv Clinical Hospital of Kyiv.

Inclusion and Exclusion Criteria

The patients diagnosed with chronic rhinosinusitis after surgical treatment (endonasal endoscopic maxillotomy, septoplasty, Vasotomy of the inferior turbinates), aged 18 to 60 years, and the presence of a signed informed consent form to participate in the study were taken into consideration. While, patients with the following conditions pregnancy, taking antibacterial agents and immunosuppressants for 3 months, alcoholism, drug addiction, mental disorders, decompensated forms of diseases, oncological diseases, inhalation allergy, chronic polyposis rhinosinusitis, and surgical interventions on the nasal cavity in the past, were excluded from the study.

Determination of Nitrite Content and NO-Synthase Activity

We determined the total activity of NO-synthase (gNOS), as well as the activities of inducible (iNOS) and constitutive (cNOS) isoforms of NO-synthase, in the sinonasal fluid (Akimov & Kostenko, 2016; Yelins'Ka *et al*., 2019). The increase in nitrite concentration following the incubation of 0.2 ml of the chosen research material in 2.9 ml of an incubation solution containing: was used to measure the total activity of NO-synthase. 2.5 ml of a solution buffered with 0.2 M Tris (pH = 7.4), 0.3 ml of an aqueous solution containing 320 mM L-arginine, and 0.1 ml of an aqueous solution containing 1 mM NADPH+H [162]. The calculation was made according to the formula:

$$\text{Total NOS activity} = (E2\text{-}E1) \times 2057/N;\ \mu\text{mol/min}\times\text{g of protein}, \quad (1)$$

where E1 is the optical density before incubation, E2 is the optical density after incubation, and N is the concentration of total protein (g/l).

After incubating 0.2 ml of the selected material for research in 3.1 ml of an incubation solution containing the following, the increase in the concentration of nitrites was used to determine the activity of constitutive isoforms: 2.5 ml of a solution of 0.2 M Tris buffer (pH = 7.4), 0.3 ml of an aqueous solution containing 320 mM L-arginine, 0.1 ml of an aqueous solution containing 1 mM NADPH+H, and 0.2 ml of an aminoguanidine hydrochloride solution containing 1% (a specific inhibitor of iNOS). Incubation of the studied sample lasted 60 min (Yelins'Ka *et al*., 2019). The calculation was made according to the formula:

$$\text{Activity of constitutive NOS isoforms} = (E2\text{-}E1) \times 1028.5/N;\ \mu\text{mol/min}\times\text{g of protein}, \quad (2)$$

Where E1 is the optical density before incubation, E2 is the optical density after incubation, and N is the concentration of total protein (g/l).

Determination of Arginase Activity

The activity of arginase was assessed by the increase in the concentration of L-ornithine, which was determined by the colored product formed in the reaction with the Chinard reagent in Khramov's modification, after incubation in a phosphate buffer medium (pH=7.0) containing L-arginine (Khramov & Listopad, 1973; Akimov & Kostenko, 2016).

By adding 0.1 ml of the research material to a medium containing 0.5 ml of 0.2 M phosphate buffer solution, the initial concentration of L-ornithine was determined. After that, 1 milliliter each of glacial acetic acid and ninhydrin reagent was added, and the mixture was boiled for one hour in a water bath. The mixture was then centrifuged at 3000 rpm with 1 ml of 20% trichloroacetic acid added. 40 min. Then 1 ml of the supernatant was photometered at a frequency of 490 nm against water.

By adding 0.1 milliliters of the chosen research material to a medium containing 0.5 milliliters of 0.2 M phosphate buffer solution and 0.2 milliliters of 24 mM L-Arginine solution, the final concentration of L-ornithine was determined. The test tubes were then sealed and left to incubate for 20 hours. at t=37 ℃. After that, 1 milliliter each of glacial acetic acid and ninhydrin reagent was added, and the mixture was boiled for one hour in a water bath. The mixture was then centrifuged at 3000 rpm with 1 ml of 20% trichloroacetic acid added. 40 min. Then 1 ml of the supernatant was photometered at a frequency of 490 nm against water.

Arginase activity was calculated according to the formula: Arginase activity = 1000×(Final concentration of L-ornithine – Initial concentration of L-ornithine)/(1200×N) where N is the protein concentration in g/l. Units of measurement are μmol/min×g of protein.

Ethical Approval

According to the requirements of the Tokyo Declaration of the World Medical Assembly, the International Recommendations of the Helsinki Declaration of Human Rights, the "Council of Europe Convention on Human Rights and Biomedicine," the Orders of the Ministry of Health of Ukraine, the "Basics of Ukrainian Legislation on Health Care" as amended, the Code of Ethics of a Doctor of Ukraine and the Code of Ethics of a Scientist of Ukraine, current legislation, and confirmed by the permission of the Ethics Committee Bogomolets National Medical 153, dated 29 November 2021.

Statistical Analysis

Factual handling of the information was completed utilizing Measurable programming EZR v. 1.55 (graphical UI for R factual programming adaptation 4.1.2, R Starting point for Measurable Processing, Vienna, Austria).

Results and Discussion

The results showed no statistically significant difference in age and gender was found between groups of patients. The levels of iNOS, cNOS, gNOS, and arginase showed non-significant differences among experimental and control groups one day before surgery **(Table 1)**. Whereas, on the 3rd day after surgical treatment, the level of iNOS (P=0.01), gNOS (P=0.017), and Arginase (P=0.02) showed significant differences among the experimental and treatment group. However, there was a non-significant difference (P=0.114) in cNOS levels among the experimental and treatment groups on the 3rd day after surgical treatment as shown in **Table 1**.

The results of iNOS, cNOS, gNOS, and arginase levels on the 10th day after surgical treatment showed significant differences among the experimental and treatment groups whereas, a similar pattern was observed on the 28th day after surgical treatment **(Table 1)**. The level of iNOS was first increased on the 3rd day after surgery and then decreased on the 10th and 28th days after surgery when compared to the levels one day before surgical treatment in both experimental and control groups. A similar, trend was observed for the levels of gNOS in experimental and control groups. However, there was a slight decrease in the level of cNOS after surgical treatment in the experimental group while the level of cNOS increased in the control group. Whereas, the level of arginase increased at 3rd day after surgical treatment in both the experimental and control groups **(Table 1)**.

Chronic rhinosinusitis is a serious social, economic, and health issue (Adouly *et al*., 2017; Wahid *et al*., 2020). According to estimates, the prevalence and incidence of chronic rhinosinusitis are increasing and are on par with those of diabetes and heart disease (Bachert *et al*., 2021). The major objective of treating chronic rhinosinusitis is to keep the patient's quality of life and sinus symptoms under control. As a result, one of the key factors in deciding whether to start or intensify therapy for chronic rhinosinusitis is symptom management (Fokkens *et al*., 2012). Even though NF-kB is inhibited by N-acetylcysteine, the operational harm that takes place in our experiment is a source of DAMPs and can activate the transcription factor AP-1, which enhances the production of iNOS (Jang & Surh, 2005).

Therefore, the experimental group's increased iNOS activity on the third and 28th days of the experiment may be related to the effect of N-acetylcysteine on profibrotic macrophages and their release from the control of transforming growth factor. By decontaminating damaged cells caused by surgical intervention, macrophages can effectively perform their function. According to research by Senior *et al*. (1998), patients' subjective outcomes improved after functional endoscopic sinus operations. With the proper postoperative care, this progress can be kept up over time. The results of previous surgeries were also discovered to have an impact. This study found that pre-lockdown total scores for those who had undergone two procedures were higher than post-lockdown total scores. However, it is still unknown if this is due to the disease's more severe and chronic spectrum or if the surgeon could improve outcomes by managing other factors.

NO is a key modulator of the airways, and its connection to chronic rhinosinusitis is of great interest. Under the bearing of inducible NO synthase, it is generally produced by incendiary cells and nasal and bronchial epithelial cells (Duong-Quy *et al*., 2017). The high concentration of NO, which may reach up to 23.000 ppb in the paranasal sinuses (Vlad *et al*., 2015), which is a component of the host's first-line defenses, ensures an antibacterial, antiviral, and antifungal action. Low levels of NO have been linked to decreased mucociliary transport, and NO upregulates ciliary motility to enhance mucociliary clearance (Nawroth *et al*., 2020). This is important because NO can have an anti- or pro-inflammatory effect depending on the type and stage of airway inflammation, its local concentration, and the individual's response (Vlad & Albu, 2019). It has been demonstrated that the respiratory tract's T helper type 1 (Th1) and type 2 (Th2) immunologic responses are both influenced by NO (Ibiza & Serrador, 2008; He *et al*., 2023).

Table 1. The levels of iNOS, cNOS, gNOS, and arginase were measured before surgery in patients with chronic rhinosinusitis.

Parameters	Experimental group	Control group	P-value
One day before surgical treatment (Functional endoscopic sinus surgery, FESS)			
iNOS	2.14(1.59-2.44)	1.87(1.48-2.27)	0.071
cNOS	0.11(0.09-0.12)	0.10(0.097-0.12)	0.486
gNOS	2.24(1.71-2.54	1.97(1.59-2.43)	0.087
Arginase	0.32(0.24-0.52)	0.26(0.23-0.50)	0.115
3rd day after surgical treatment (FESS)			
iNOS	2.44(2.24-2.76)	2.23(1.59-2.54)	0.01
cNOS	0.110(0.098-0.121)	0.11(0.110-0.113)	0.114
gNOS	2.54(2.34-2.88)	2.36(1.71-2.65)	0.017
Arginase	0.33(0.32-0.39)	0.44(0.33-0.45)	0.002
10th day after surgical treatment (FESS)			
iNOS	1.70(1.37-1.73)	1.15(0.82-1.72)	0.008
cNOS	0.103(0.091-0.114)	0.115(0.113-0.126)	0.002
gNOS	1.81(1.45-1.82)	1.27(0.92-1.84)	0.039
Arginase	0.41(0.39-0.65)	0.65(0.41-0.67)	0.004
28th day after surgical treatment (FESS)			
iNOS	0.92(0.89-1.1)	0.87(0.61-1.05)	0.006
cNOS	0.101(0.099-0.122)	0.118(0.104-0.124)	<0.001
gNOS	1.02(0.99-1.22)	0.98(0.73-1.16)	0.01
Arginase	0.61(0.60-0.70)	0.75(0.60-1.00)	0.003

Interleukin (IL)- 2 and interferon-gamma (INF-) are emitted by Th1 cells, which are connected to non-eosinophilic provocative problems and high phagocytic action (Fahy, 2015). Th2 are essential for allergies; They produce IL-4, IL-5, and IL-13 as part of their immune response, which attract and activate mast cells, basophils, and eosinophils and cause goblet cell hyperplasia in the airway mucosa (Koyasu & Moro, 2011; Fahy, 2015).

In the ongoing examination, the higher nitrite content on the third day of the trial is an immediate consequence of the raised iNOS action right now. On day 28, the amount of nitrites did not change in a statistically significant way. This suggests that the mucosal

membrane's antibacterial defenses had been strengthened by high iNOS activity and that the overproduction of nitric oxide had been effectively utilized. Patients with chronic rhinosinusitis have low NO levels. Its decay is in accordance with the seriousness of the condition, and an ascent in fixation was seen following the organization of treatment measures (Taruya *et al*., 2015; Vlad *et al*., 2015).

A few reasons have been proposed to make sense of the diminished degrees of breathed out and nasal NO found in people with persistent rhinosinusitis: (1) obstruction of the sinus ostium and an increase in mucosal absorption; (2) harm to the NO-producing sinus mucosa as a result of an increase in the production of cytotoxic agents in chronic inflammation; or 3) decreased iNOS expression as a result of specific cytokines that are present in the sinus mucosa of people who have chronic rhinosinusitis. IL-4, IL-6, and TGF- are among these cytokines (Dabholkar *et al*., 2014).

The two isoforms of arginine are arginine I (cytosolic) and arginine II (mitochondrial) (Morris, 2009). Just a single other review, directed by Taruya *et al*. (2015), has also looked into the role of arginase in persistent sinusitis. There is high proof that expanded aspiratory arginase 1 action assumes a significant part in propagating or potentially potentiating eosinophilic fiery lung sickness, thought to be through the lessening of NO creation (Pera *et al*., 2014). It hasn't been demonstrated to be as important in the pathophysiology of CRS as it has in the lower airways. Similar findings were made by Taruya *et al*. (2015) in their investigation of 45 cases. Arginase 1's role in asthma may be more closely tied to other, non-NO-related processes that are controlled by this enzyme.

On days 3, 10, and 28, there was more arginase activity in the control group. This may be due to the influence of a high concentration of transforming growth factor-, which can boost the production of this enzyme, in the postcovid state (Kupani *et al*., 2021; Oronsky *et al*., 2023). Its decreased activity in the experimental group is related to N-acetylcysteine's capacity to block signaling through the TGF- β pathway (Yazaki *et al*., 2021). According to subgroup analysis, patients without polyps had considerably higher arginase 2 readings, which was in line with Taruya's findings (Taruya *et al*., 2015), It showed a link between higher ARG2 expression and lower NO levels exhaled. As a result of reduced NO synthesis caused by elevated ARG2, there is induction of Th1 differentiation, non-eosinophilic inflammation, and macrophage activation, all of which eventually limit NO release because there is no substrate. The CRSwNP group had higher ARG2 expression than the CRSsNP group, but the control group had lower expression. Th1 proinflammatory cytokines and a cellular infiltration of neutrophils, macrophages, and lymphocytes, are present in both types of CRS (Lee & Lane, 2011). An eosinophilic inflammatory infiltration and a preponderance of Th2-type cytokines are characteristics of CRSwNP (Kato, 2015; Shah *et al*., 2016). Superior NO levels promote Th2 proliferation, IgE generation, and eosinophil recruitment, as was previously indicated. It is highly likely that low to moderate levels of ARG2 partly restrict NO synthesis, rendering it incapable of maintaining a protective role and allowing NO to build up to levels sufficient to encourage differentiation of Th2 Numerous studies suggest that CRSwNP's low nasal nitric oxide (nNO) concentration is due to ostiomeatal blockage (Jeong *et al*., 2014). An obstruction in the sinus may cause an increase in NO, which stimulates the Th2 immune system excessively and triggers a series of local responses that eventually prolong eosinophilic inflammation. In this context, Lee *et al*. (2015) recently demonstrated that following endoscopic sinus surgery (ESS), nNO levels of CRSwNP patients considerably rose while being unchanged in CRSsNP. Following ESS in CRSwNP, nNO is currently thought to be a measure of sinus mucosal health. However, our results are more in favor of ARG2 being involved in non-Th2-mediated inflammation in CRS pathogenesis.

Conclusion

The findings of this study provide insight into the causes of chronic rhinosinusitis without polyps, the function of NO and iNOS, and the possible therapeutic advantages of N-acetylcysteine in treating this illness. According to the research, iNOS and arginase activity are considerably changed in chronic rhinosinusitis patients, demonstrating their participation in the disease's inflammatory processes. Additionally, topical N-acetylcysteine treatment appears to have a positive impact on the control of NO production and arginase activity, which may help to reduce inflammation and enhance clinical results.

These findings underscore the significance of taking the L-arginine-nitric oxide pathway into account when examining the pathophysiology of chronic rhinosinusitis and point to N-acetylcysteine as a potentially effective supplementary therapy for this illness. To fully understand the underlying processes and to investigate the potential of N-acetylcysteine in a wider context, including its implications for treating other respiratory disorders like COVID-19, more study is necessary. The results of this study may open the door to more specialized and successful therapy approaches for individuals with chronic rhinosinusitis without polyps.

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