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Investigation of Oxidative Stress and Antioxidant Pathways in Nasal Polyp Tissue: Peroxynitrite and Malondialdehyde Compared to NF-E2-related factor 2, Kelch-like ECH-associated protein one and Glycogen Synthase Kinase-3ß

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ABSTRACT

Objective: Nasal polyps are benign mucosal lesions with multifactorial causes that grow into the nasal cavity and are associated with inflammation. This study aims to investigate whether Nrf2, Keap1, GSK-3ß, Peroxynitrite, and malondialdehyde may be used as biochemical markers to determine the relationship between oxidative stress and nasal polyps. The goal is to explore the etiology of nasal polyps and contribute to the literature for a better understanding of the inflammatory pathophysiology of nasal polyps, ultimately leading to the development of new therapeutic approaches.

Materials and Methods: A total of 94 patients aged between 12 and 65 years who underwent a surgical operation for polyps (n = 49, case group) and septoplasty (n=45, controls) between February and September 2022 at the Department of Otorhinolaryngology, Faculty of Medicine, Gaziantep University were included in the study. Tissues taken from the polyp and the inferior turbinate in the case and control groups, respectively, were homogenized at the biochemistry laboratory and investigated using the ELISA method to compare the Nrf-2, Keap1, GSK-3ß, malondialdehyde, and peroxynitrite levels.

Results: Consistent with the study hypothesis, Nrf2 levels were lower, and Keap1 levels were higher in the case group, although the difference was not statistically significant. Although studies have reported increased levels of GSK-3β in chronic rhinosinusitis, they were statistically lower in polyps. This may be associated with the complexity of the GSK-3β network or the adequacy of Keap1 alone for Nrf2 inhibition. Peroxynitrite and malondialdehyde (MDA) levels are indicators of oxidative stress.

Conclusion: Nrf2, Keap1, GSK-3ß, MDA, and Peroxynitritetrite may be involved in the aetiology of nasal polyps based on the study's results. Keap1 and GSK-3ß, Nrf2 and Nrf2 module, actors which regulate oxidative stress, played a role in the pathophysiology of nasal polyps in combination with Peroxynitritetrite and malondialdehyde, according to the study findings. Potential treatments for nasal polyps are better understood through more extensive and well-matched studies.

Keywords: Gsk-3ß, Keap 1, Malondialdehyde, Nasal Polyp, NRF2 Peroxynitrite, Oxidative Stress.

INTRODUCTION

Nasal polyposis (NP) is a complex inflammatory condition with several contributing factors. The chronic adaptive immune response observed in NP is likely the result of defects in the essential functions of the airway epithelium, in combination with elevated proinflammatory cytokines and chemokines (1). While the NP model provides investigators with a unique opportunity to observe an ongoing inflammatory response in a human system, the lack of animal models and reliance on descriptive and correlative data have limited the ability to study the mechanisms of the pathogenesis of this disease (2). As a result, there is a paucitlackta demonstrating the necessity or sufficiency of any of these potential drivers of NP. Therefore, it is essential to conduct clinical studies with new drugs targeting specific cytokines, cytokine receptors, or signalling molecules in patients (1).

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A robust epidemiologic dataset is also required to aid mechanistic studies in NP research. Given the high prevalence, costs, and morbidity associated with this disease, there is a great need for continued research that could facilitate the development of novel therapeutic strategies to improve the treatment of patients with this disease.

NF-E2-related factor 2 (Nrf2) is an anti-stress system that protects cellular homeostasis through interactions between Nrf2 and Kelch-like ECH-associated protein 1 (Keap1). First described by Moi et al., Nrf2 belongs to the Cap-n-Collar family of basic leucine zipper proteins (3), is a transcription factor that promotes the expression of the globin gene, and has also been described as a primary sensor of oxidative stress in cells (4). Nrf2 protein stability is phosphorylated by glycogen synthase kinase 3β (GSK-3β) and regulated by two E3 ubiquitin ligase adaptors, including Keap1 is one of them (5). In the normal range, the Nrf2/Keap1 pathway is an antioxidant protective mechanism; however, when it is insufficiently or over-synthesized, it prevents damaged cells from undergoing apoptosis and leads to tissue hypertrophy, cancer, and chronic diseases (6, 7).

Malondialdehyde (MDA), one of the degradation products of lipid peroxidation, can be used as an indicator of free oxygen radical damage in healthy tissues (8). Non-free radPeroxynitritetrite is a potent and directly toxic oxidizing agent and marker of oxidative stress in the body (9). Oxidative stress, caused by an imbalance between the rates of oxidation and antioxidant defense mechanisms, is directly linked to chronic inflammation and can lead to various physiological and pathological conditions. Studies have shown that Oxidative stress is high in nasal polyposis and plays a role in the etiopathogenesis of NP (1, 10).

The primary aim of this study is to bridge the current gaps in NP research by investigating the roles of oxidative stress and antioxidant defence mechanisms in NP pathogenesis. Despite considerable research, the necessity and sufficiency of potential drivers of NP remain inadequately demonstrated. This study explores the relationship between NP and specific biochemical markers, such as Nrf2, Keap1, GSK-3ß, malondialdehyde, and peroxynitrite, to elucidate their potential as indicators in NP evaluation. Furthermore, it seeks to prompt new pharmacotherapeutic investigations tailored towards targeting these markers, thus contributing to the development of novel therapeutic strategies for patients with NP. This approach directly addresses the current research gap by providing a detailed analysis of the oxidative stress markers and their role in the etiopathogenesis of NP, thereby offering a new perspective on developing and treating this chronic condition.

MATERIAL and METHODs

Patient Selection

Patients aged 12-65 and opera who underwent surgery for nasal polyps at Gaziantep University Şahinbey Research and Application Hospital between February and September 2022, with no known chronic disease and no history of drug use, were included in the group consisting of polyp tissue samples from 49 patients who underwent surgery for nasal polyps. Patients undergoing surgery for nasal polyps were selected to establish a clear demarcation between affected and control tissues, enabling a direct comparison of oxidative stress markers. Control specimens were acquired from the lower turbinate mucosa tissue samples of 45 patients undergoing surgery due to anatomical defects. Nrf-2, Keap1, GSK-3ß, malondialdehyde, and peroxynitrite levels were compared. This study was approved by the local ethics committee (decision number: 2021/395). All participants and their parents signed an informed consent form.

Inclusion and Exclusion Criteria for the Research

Inclusion criteria for the study were: volunteering to participate, ages 12 -65, being not pregnant or suspected of being pregnant, having no history of drug use due to nasal polyps in the last four weeks, having no additional diseases besides nasal polyps (for the case group), and conditions other than pathology (for the control group).

The exclusion criteria were alcohol or substance addiction, anti-inflammatory or antioxidant drug use, presence of acute or chronic systemic disease, pregnancy or suspected pregnancy, and unwillingness to participate in the study.

Collection of Tissue Samples and Laboratory Study

Nasal endoscopy was used to collect tissues under general anesthesia and sterile conditions. An IKA Ultra Turrax T25 homogenizer device was used to mechanically homogenize one tissue volume and nine physiological saline volume homogenization processes using the Branson Sonifier 150 device for 20 s. The mixture was then centrifuged for 1 at 2,000 rpm in a cold environment. The supernatant was separated, placed in Eppendorf tubes of 2 ml volume, and stored at -80 C. The ELISA method was utilized for its sensitivity and specificity in quantifying the targeted biochemical markers. The ELISA method measured NRF2, Keap 1, GSK3ß, malondialdehyde, and peroxynitrite levels. Nrf2, GSK3B, Keap1, MDA, and peroxynitrite markers were analyzed using a BT-LAB ELISA KIT. The samples were evaluated using an ELISA reader by applying the experimental procedure to the relevant parameters. As a result, Nrf2, GSK3B, and Keap1 parameters were calculated as ng/g wet tissue, and MDA and peroxynitrite parameters as µM/g wet tissue.

Statistical Evaluation

The conformity of numerical variables to a normal distribution was compared using the Shapiro–Wilk test, Student's t-test was used to compare normally distributed variables between the two groups, and the Mann-Whitney U test was used to compare non-normally distributed variables between the two groups. ANOVA and LSD tests were used to compare normally distributed variables in the three groups, and Kruskal–Wallis and Dun. Kruskal's test compared non-normally distributed variables among the three groups. The relationships between non-normally distributed numerical variables were calculated using Spearman's correlation coefficient. Associations between categorical variables were tested using the chi-squared test. Multiple linear regression was used to evaluate the effects of the variables on biochemical parameters and the presence of disease. The analysis was conducted using SPSS 22.0, Windows version. Statistical significance was set at $P < 0.05$.

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RESULTs

This study was performed on age- and sex-matched patients. An overall sample size of 94 individuals aged 12-65 years was used in this study; 49 patients underwent surgery for nasal polyps and 45 patients underwent surgery for septal deviations (SD) as the control group. None of the participants had any known acute or chronic diseases that could affect the oxidative stress parameters or antioxidant use. Among the participants in the study group, 11 (22.4%) had allergic asthma along with nasal polyposis, while none in the control group had this condition. No significant difference was found between the groups regarding sex and smoking status, whereas age differed significantly (p=0.02). The general characteristics of the study participants are presented in **Table1** in detail.

While some of the patients in the study underwent functional endoscopic sinus surgery for the first time, others underwent repeated nasal polyp operations. A description of the pathological diagnosis of the specimen and data regarding the recurrence of nasal polyposis surgery are presented in **Table2**.

A statistically significant difference was found in GSK-3ß and MDA levels: MDA levels were significantly higher (p=0.041), and NRF2 and GSK-3ß values were significantly lower in the case group (p=0.049 and 0.001, respectively). The Keap1 and peroxynitrite levels were not statistically different (**Table3**).

No statistically significant differences between allergic and inflammatory polyp results were observed in the pathology report regarding Nrf2, Keap1, GSK-3B, malondialdehyde, and peroxynitrite values (**Table4** Pearson's correlation was used to compare whether there was a correlation between Nrf2, Keap1, GSK-3ßPeroxynitritetrite in the study group, and a weak positive statistically significant correlation was found between Nrf2 and GSK-3ß (r=0.337, p=0.018) and MDA (r=0.362, p=0.011). A weak negative correlation was found between Nrf2Peroxynitritetrite (r= -0.367, p=0.009). A positive correlation was observed between Keap1 and MDA levels (r=0.385, p=0.006). No correlation was observed between any other parameters.

We compared Nrf2, Keap1, GSK-3ß, malondialdehydePeroxynitritetrite levels with age to determine if they were influenced by age and found no statistically significant difference between age and Nrf2(r=-0.034, p=0.817), Keap1(r=0.168, p=0.249), GSK-3ß(r=0.027, p=0.856), malondialdehyde (r=-0.021, p=0.885), and peroxynitrite (r=0.12,0.411) levels.

As part of the study, we investigated whether Nrf2, Keap1, GSK-3ß and oxidative stress parameters in the nasal polyp tissues were affected by age, sex, and smoking and found that age (r=0.003, p=0.323), sex (r= -0.137, p=0.891), and smoking (r=0.340, p=0.734) had no statistically significant effect on Nrf2, Keap1, GSK3B, MDA, and peroxynitrite values.

Lund-Mackey staging was performed using CT to evaluate the prevalence of nasal polyps. The Lund-Mackey staging system has a score range of 0-24; with higher scores indicating a higher prevalence of nasal polyps. Scores ranged from 8 to 24 in the present study. The patients were divided into three groups based on their scores: the first group had 8-13 points, the second group had 14-19 points, and the third group had 20-24 points. The prevalence of polyps was mild in the first group, moderate in the second, and advanced in the third.

The groups were compared for Nrf2, Keap1, GSK3B, MDA, and peroxynitrite levels. A statistically significant difference was observed between the groups in peroxynitrite levels (p=0.023); however, no significant difference was detected in Nrf2, Keap1, GSK3B, and MDA levels.

While the mean peroxynitrite level was the lowest in the 1st group, it was the highest in the 3rd group, and the difference was statistically significant. Furthermore, in multiple comparisons between the peroxynitrite groups, the difference between the 1st and 3rd groups was statistically significant (p=0.006).

A significant difference was found between the control and patient groups in terms of the peroxynitrite value in the nasaregardingp=0.076). Peroxynitrite levels were the lowest in the control group and increased directly with polyp prevalence.

A significant difference was observed between the control and patient groups in terms of Nrf2 expression in nasal tissue (p<0.05). Nrf2 levels were the highest in the control group and increased directly with polyp prevalence.

Although the difference was not statistically significant, the mean Keap1 and MDA values in the nasal tissues of the control group were lower than those of the polyp groups (p=0.092 and p=0.041, respectively). Mean Keap1 and MDA values were lowest in the control group and increased directly with polyp prevalence (**Table5**).

Table 1. Demographic features of study participants.

	Age, mean ± SD, years	Male/Female n (%)	Smokers (%)
Study group	29.96 ± 11	32 (55.4)/ 17 (34.6)	16 (32.6)
Control group	36.69 ± 13	33 (73.4) /12 (26.6)	22 (48.8)
p	**0.02**	0.67	0.45

*Student paired test, p>0.05

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Table 2. The pathologic diagnosis of the specimen and the nasal polyposis surgery recurrency

		n=49	%
Operation	FESS	36	73,4
	Rev.FESS	13	26,6
Pathology	Allergic polyposis	25	51
	Imflamatuary poliposis	24	49

FESS: first time for functional endoscopic sinus surgery, Rev.FESS: recurrent functional endoscopic sinus surgery.

Table 3: Assessment of Nrf2, Keap1, GSK-3B, Malondialdehyde and Peroxynitrite levels in Cases and Controls.

	Study group (n=49)	Control group (n=45)	P
NRF2 ng/gr	18,17 ± 7,67	21,37 ± 7,92	0,049
KEAP1 ng/gr	620,18 ± 227,5	561,38 ± 280,45	0,092
GSK3B ng/gr	366,03 ± 166,33	482,4 ± 136,21	0,001
MDA µM/ gr	24,83 ± 7,51	21,53 ± 7,96	0,041
Peroxynitrite (µM/ gr wet tissue)	7,4 ± 3,95	5,85 ± 2,61	0,076

Student t test *p<0,05
NRF2: NF-E2-related factor, KEAP1: Kelch-like ECH-associated protein, GSK3B: Glycogen Synthase Kinase, MDA: Malondialdehyde.

Table 4: Comparison of Nrf2, Keap1, GSK-3B, Malondialdehyde and Peroxynitrite levels based on pathology results.

	Allergic polyp (n=25)	Inflammatory Polyp (n=24)	P
NRF2 ng/gr	17,36 ± 8,04	19,01 ± 7,35	0,582
KEAP1 ng/gr	588,99 ± 219,92	652,67 ± 235,36	0,327
GSK3B ng/gr	348,84 ± 162,77	383,93 ± 171,58	0,466
MDA µM/ gr	24,14 ± 8,46	25,54 ± 6,48	0,519†
Peroxynitrite (µM/ gr yaş doku)	7,62 ± 4,17	7,18 ± 3,79	0,660

Table 5: Comparison of the groups representing the prevalence of nasal polyps in terms of Nrf2, Keap1, GSK3B, MDA, and Peroxynitritetrite levels.

	Group 1 (n=12)	2.Grup (n=10)	3. Grup (n=27)	*P
NRF2 ng/gr	21,47 ± 6,39	19,44 ± 9,1	16,24 ± 7,29	0,130
KEAP1 ng/gr	564,89 ± 144,52	650,59 ± 293,35	633,49 ± 234,47	0,622
GSK3B mg/gr	397,33 ± 189,53	435,83 ± 179,11	326,26 ± 144,24	0,156
MDA µM/ gr	24,99 ± 6,53	23,69 ± 9,73	25,17 ± 7,26	0,869
Peroksinitrite (µM/ gr)	5,28 ± 2,16	6,48 ± 2,7	8,69 ± 4,5	0,023

Student t test *p<0,05
Group 1: mild prevalence of polyps, Group 2: moderate prevalence of polyps, Group 3: severe prevalence of polyps

DISCUSSION

This study investigated the relationship between nasal polyposis and oxidative stress. Peroxynitrite levels, which are accepted as a strong oxidant; MDA levels, which are an indicator of oxidative stress in the body; Nrf2 levels, which act as a protective mechanism against oxidative stress; Keap1 and GSK-3ß levels, which are very important in controlling Nrf2 activity, were studied in tissue samples taken from 12-65 years old patients with a diagnosis of NP and age- and sex-matched control patients. We aimed to understand whether they can be used as supportive biochemical markers to evaluate NP formation and to commence new pharmacotherapeutic studies.

The prevalence of nasal polyps in the literature has been estimated to range between 0.5 and 4%, depending on the population. NP is more prevalent in males, and its incidence increases with age (11). Etiological factors such as genetics and racial characteristics contribute to different population rates (12). In agreement with the literature, our study included 49 male patients with NP, with a median age of 36 ±13 years.

Several studies have shown that inflammatory cells are closely associated with histopathological abnormalities in NP. At the site of inflammation, neutrophils produce reactive oxygen species (ROS), which exert bactericidal effects.

When activated, neutrophils migrate to the site of inflammation. Increased oxidative stress may result in cell damage, tissue damage, and chronic diseases when ROS production and antioxidant defences are disrupted (13).

Our study found a statistically significant difference between the case and control groups regarding Nrf2 GSK-3ß and MDA values. Consistent with the literature, Nrf2 levels were low and Keap1 and peroxynitrite levels were high. These results support the inadequacy of antioxidant mechanisms and high oxidative stress in the formation of nasal polyps.

Studies investigating ROS and antioxidants in nasal polyposis provide strong evidence that oxidative stress is involved in the pathogenesis of NP, and MDA levels are higher in nasal polyp tissue, suggesting that oxidative stress plays a role in the formation of nasal polyps (14-16). In our study, MDA levels were also significantly higher in the NP group, which is consistent with the literature. The fact that there was an increase in nasal polyp tissues supports the hypothesis that oxidative stress may be effective in forming nasal polyps.

Peroxynitrite is a highly reactive form of peroxynitrite that causes oxidative tissue damage. Peroxynitrite production is low under normal physiological conditions, and endogenous antioxidants buffer its oxidative action. Increased peroxynitrite levels may play a role in the pathophysiology of various diseases, such as DNA damage, vascular diseases, ischemia-reperfusion injury, circulatory shock, inflammation, pain, and neurodegeneration (17, 18). Peroxynitrite has been detected in nasal polyp tissues, indicating that it may play a role in the pathophysiology of nasal polyps (19, 20). High-level peroxynitrite in nasal polyp tissue is thought to play a role in the pathophysiology of nasal polyps by disrupting the nasal epithelial barrier, causing inflammation, increasing the number of eosinophils, and causing metaplasia in the nasal polyp mucosa (20). Consistent with the literature, the mean peroxynitrite value in our study was higher than that of the control group, supporting the role of oxidative stress in NP formation.

Nrf2 is induced by GSK-3β inhibitors (137–139). Low Nrf2 activity can lead to decreased antioxidant capacity, whereas high Nrf2 can cause detrimental consequences, such as epithelial cell hyperplasia and the inability of some cell types to differentiate correctly. Disruption of the Nrf2 control mechanism (excessive increase or decrease) leads to several conditions, such as cancer, neurodegenerative disease, cardiovascular disease, diabetes, chronic obstructive pulmonary diseases, autoimmune diseases, and inflammation (138).

Research on the effects of Nrf2, Keap1 and GSK-3ß on nasal polyps is limited, but there have been studies on chronic rhinosinusitis. According to a survey conducted on the sinonasal mucosa, low levels of Nrf2 stabilize the sinonasal epithelial barrier function and reduce rhinosinusitis by inhibiting Keap1 (180). According to another experiment, eosinophilic sinonasal inflammation becomes more severe when the Nrf2 pathway is disrupted (184). Antioxidant pathways may be an effective therapeutic target for chronic rhinosinusitis because they play a crucial role in determining susceptibility to eosinophilic inflammation. Our study indicated that Nrf2 levels were lower, and Keap1 levels were higher (though not statistically significant) in the NP group, which is consistent with the literature data. The results of our study suggest that oxidative stress may contribute to the development of NP

GSK-3β inhibits Nrf2 and increases oxidative stress when Nrf2 levels increase. To our knowledge, no study has evaluated the relationship between NP formation and GSK-3β in nasal polyp tissue; however, studies have been conducted in patients with chronic rhinosinusitis. Abnormal GSK3β expression has been detected in the nasal mucosa of patients with chronic rhinosinusitis (21, 22). GSK-3ß has been demonstrated to be one of the most essential regulatory mechanisms, and Nrf2 has been shown to reduce Nrf2 levels in many studies (23, 24).

In our study, the mean GSK3β level was significantly lower in the GSK-3ß Theted to cause high Nrf2 levels; however, Nrf2 levels were low in the case group. In other words, our results on GSK3β are inconsistent with the literature. This may be because of the complexity of the GSK-3β network or the adequacy of Keap1 alone for Nrf2 inhibition.

We found a negative correlation between Nrf2 and peroxynitrite levels and a positive correlation between Nrf2 and Gsk-3ß levels. The negative correlation between peroxynitritetrite and Nrf2 is consistent with literature. However, recent studies have suggested a positive correlation between the two (25, 26).

We divided the patients with polyps into three groups based on the Lund-Mackey (76) staging system. Peroxynitrite was lowest in the first group and highest in the third group, and the difference was statistically significant. The mean peroxynitrite value was lowest in the control group and increased directly with polyp prevalence, supporting oxidative stress. The increase in peroxynitrite value concurrent with the prevalence of polyps supports our hypothesis and the literature data that it may be influential in polyp formation.

Similarly, the mean Nrf2 values of the control group were higher than those of the polyp groups; Nrf2 with media was the highest in the control group and decreased inversely with polyp prevalence, supporting antioxidant weakness. This result supports the hypothesis that, while the prevalence of polyps increases, antioxidant mechanisms are weakened or insufficient, and oxidative stress increases.

Finally, the mean Keap1 and MDA values of the control group were lower than those of the outer appearance of the polyp, and the mean Keap1 and MDA values were the lowest in the control group and increased gradually with polyp prevalence, sparing oxidative stress. Once again, our results support the hypothesis that oxidative stress may be effective in polyp formation.

Although there are reports in the literature that age, sex, and smoking affect Nrf2, Keap1, GSK-3ß, malondialdehyde, and peroxynitrite levels, and many studies have shown that smoking increases oxidative stress, our study found no statistically significant difference in the comparisons of peroxynitrite separately for age, sex, tobacco and Nrf2, Keap1, GSK-3ß, malondialdehyde (6, 7, 12, 25). Further studies may be necessary to verify this information.

Studies show that Oxidative stress plays a vital role in the pathophysiology of allergic asthma (1, 15, 19, 26). Our study did not find a statistically significant difference in Nrf2, Keap1, GSK-3ß, malondialdehyde, or peroxynitrite levels between patients between patients with and without allergic asthma ith and without allergic asthma. Therefore, it is possible that permanent nasal mucosal damage occurred.

Based on the study results, oxidative stress is increasing in NP; Nrf2, one of the most important signalling pathways that can initiate protective mechanisms, lacks the ability to balance the high load of oxidative stress and annotates insufficient antioxidant defense systems in NP.

Straightens of the Study

To the best of our knowledge, this is the first study in which Keap1 and GSK-3ß parameters, which are the most critical components in the Nrf2 and Nrf2 signalling pathways, as well as MDA Peroxynitritetrite, were evaluated together in nasal polyp tissues.

Study Limitations

A limitation of this study may be that it was a cross-sectional study, and control values could not be seen after treatment. In addition, our study was limited to measuring Nrf2, Keap1, GSK-3ß, MDA, and peroxynitritetrite levels in tissues only. The extent to which the findings obtained from tissues with NP reflect serum levels remains unclear.

Considering our findings that Nrf2 levels are reduced, whereas Keap1, MDA, and peroxynitrite levels are elevated in nasal polyps, we propose that these markers play a significant role in the pathogenesis of NP. Further exploration of the Nrf2 pathway as a therapeutic target is warranted. Prospective studies should investigate the potential for Nrf2 activation to mitigate oxidative damage and inflammation in NP. Additionally, clinical trials exploring the efficacy of Nrf2-modulating drugs in NP treatment could offer new insights into disease management. Our research paves the way for such investigations, which may lead to innovative therapeutic approaches for patients with NP.

CONCLUSION

Among patients diagnosed with nasal polyps, Nrf2 levels were low, while peroxynitritetrite, Keap1, and MDA levels were high. Based on our data, Nrf2, Keap1, GSK-3ß, MDA, and peroxynitrite may be involved in the etiology of NP.

Understanding Nrf2 as the primary regulator of the cellular defence mechanism against oxidative stress and identifying essential proteins that regulate this pathway will allow us to understand the role of oxidative stress in the etiology of NP and guide us toward potential treatments of the disease.

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Author Contributions: HI, LSM, ST: Designed and directed the study, Literature search, Data collection, Statistics **HI:** Article writing, Final revisions. All authors reviewed the results and approved the final version of the manuscript.

Ethical approval: The present study was conducted in strict accordance with the principles outlined in the Declaration of Helsinki. Ethical approval for the study was obtained from the appropriate ethics committee..

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