

The expression of inducible nitric oxide synthase and endothelial nitric oxide synthase in the heart of smoking rats: An immunohistochemical study

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ABSTRACT

Background: Smoking is a severe health problem because it impacts the health of individuals through direct impacts on the cardiovascular system.

Study Objective: The present study aimed to investigate the expression of nitric oxide synthase isoforms in the heart of smoking rats.

Methodology: A total of 20 albino male rats were randomly assigned into two groups, the control group (N=10) and the smoking group (N=10). Smoking group rats were exposed to smoking using the smoking box for one month. Control group rats were exposed to fresh air. At the end of the experiment, all animals were terminated using ether. The heart tissues of all animals were taken and fixed in 10% formalin. Heart tissues were further processed and stained for nitric oxide synthase isoforms, Inducible Nitric Oxide Synthase (iNOS), Endothelial Nitric Oxide Synthase (eNOS), and Neuronal Nitric Oxide Synthase (nNOS) using immunohistochemistry (Indirect immunoperoxidase enzyme). The expression of NOS isoforms was calculated using Adobe Photoshop version 7.2. Antibody-stained section micrographs were analyzed using pixels. The pixels revealed the presence of the biomarker (brown) and residual tissue (blue). The relationships between the groups were computed by independent T-test. Significance was considered if p-value 0.05.

Results: The results showed that the expression of iNOS was significantly upregulated in the smoking group compared with the control group (p=0.003). The expression of eNOS was significantly downregulated in the smoking group compared with the control group (p=0.01). The expression of nNOS was not significantly varied among study groups (p=0.058).

Conclusion: This study showed that smoking negative impacts of smoking on the heart are mediated by variations in the expression of NOS isoforms, a matter that bears potential therapeutic strategies to overcome smoking outcomes.

Keywords: smoking, cigarettes, heart, Inducible Nitric Oxide Synthase (iNOS), Endothelial Nitric Oxide Synthase (eNOS), Neuronal Nitric Oxides Synthase (nNOS).

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INTRODUCTION

Smoking is a significant global health issue due to its detrimental effects on several organ systems, particularly the cardiovascular system [1]. A continuing scientific investigation is endeavoring to ascertain the intricate correlation between smoking and cardiovascular well-being [2]. Alterations in the synthesis of Nitric Oxide (NO) have been recognized as crucial factors in many mechanisms associated with cardiovascular disease in those who smoke [3]. Research has been conducted to investigate the levels of Endothelial Nitric Oxide Synthase (eNOS), Neuronal Nitric Oxide Synthase (nNOS), and Inducible Nitric Oxide Synthase (iNOS) in cardiac muscle [4]. This study aims to determine if these enzymes play a role in the development of cardiovascular disease associated with smoking. Understanding the impact of smoking on various Nitric Oxide Synthase (NOS) types in cardiac tissue is crucial for comprehending the intricate heart disorders associated with smoking [5].

Nitric oxide is a crucial signaling molecule in cardiovascular physiology that regulates myocardial contractility, promotes vasodilation, and inhibits platelet aggregation [6]. Three distinct isoforms of NOS, namely iNOS, nNOS, and eNOS, regulate the synthesis of NO in the heart in response to various physiological and pathological stimuli [7]. nNOS is predominantly present in cerebral tissues and plays a crucial role in neurotransmission, whereas eNOS is primarily located in endothelial cells and is responsible for the basal release of nitric oxide [8]. On the other hand, iNOS is present in various cell types, including cardiomyocytes, in reaction to harmful stimuli and can be activated during instances of inflammation [9].

The impact of smoking on NOS expression

Scientists investigating the impact of smoking on the regulation of NOS expression in cardiac tissue have gained further insights into the pathophysiological mechanisms behind cardiovascular illnesses in smokers [10]. Animal studies have demonstrated that prolonged exposure to cigarette smoke disrupts the NOS isoforms in the heart [11, 12]. An association has been discovered between smoking and increased production of iNOS by cardiomyocytes [13]. This mechanism results in oxidative damage and inflammation in the heart. Furthermore, alterations in the expression of Endothelial Nitric Oxide Synthase (eNOS) have been observed to result in reduced bioavailability of Nitric Oxide (NO) and impairments in endothelial function [14].

Both phenomena are linked to endothelial dysfunction and atherosclerosis generated by smoking [14]. Furthermore, there remains substantial controversy regarding the impact of smoking on the formation of nNOS in the heart [14]. Multiple studies have identified a potential correlation between smoking and cardiovascular issues, as well as alterations in cardiac function [15]. Further investigation is required to elucidate the precise mechanisms underlying the dysregulation of nNOS in individuals who smoke [16]. The isoforms of NOS in the myocardium get disrupted due to smoking, serving as a crucial trigger for the onset of heart disease [16]. To reduce the likelihood of developing heart disease and experiencing mortality as a result of smoking, it is necessary to develop targeted therapies that consider the intricate biochemical mechanisms that regulate NOS activity and translation [17]. Future research should prioritize the investigation of signaling mechanisms responsible for the alterations in NOS expression induced by smoking, as well as the development of novel pharmaceuticals capable of modulating NOS activity in individuals who smoke [18]. Additionally, the utilization of animal models to simulate smoking-induced heart disease in humans can facilitate translational research in gaining crucial insights into the efficacy of potential treatments for lowering smoking-related heart disease [19, 20].

In summary, the levels of iNOS, nNOS, and eNOS present in cardiac tissue significantly impact the regulation of NO levels and the functioning of the heart [4]. Prolonged smoking disrupts the delicate equilibrium, resulting in issues with NOS isoform regulation and cardiovascular disease [20]. Researchers aim to identify specific therapeutic approaches by elucidating the molecular mechanisms behind alterations in NOS expression induced by smoking [21, 22].

Study objectives

The main objective of this study was to explore the expression levels of nitric oxide synthase isoforms in the cardiac tissue of smoking rats using immunohistochemistry.

METHODOLOGY

Ten rats were randomly divided into two distinct groups. One group served as the control and was exposed solely to fresh air. The second group, consisting of rats that consumed one red light cigarette daily for a duration of 30 days, was exposed to red light. Rats were exposed to cigarette smoke using a specialized digital smoking box equipped with a distinctive smoking surface. Following the administration of ether to euthanize the animals, the cardiac tissues under investigation were extracted and rinsed using ordinary saline solution. Subsequently, the tissues were subjected to a 24-hours drying period in a solution of 10% formaldehyde. Following the gradual increase in alcohols used to dry the tissue, it was subsequently cleansed with xylene. To dehydrate the tissues, a sequence of increasing alcohol concentrations was employed, followed by two alterations in the amount of xylene used. The tissues underwent processing after being enveloped in pure paraffin wax. The microtome was utilized to cut sections that had a

thickness of 5 μm . Once the specimens were affixed to glass slides, they were treated with hematoxylin and eosin stain. Heart tissue was analyzed using indirect immunoperoxidase labeling to detect the presence of iNOS, eNOS, and nNOS. Heart tissue samples were processed, sectioned, and mounted on charged slides. Before immunohistochemical staining, the specimens were immersed in a solution containing 1% hydrogen peroxide for a duration of 20 minutes. This was done to inhibit the activity of the peroxidase enzyme within the cells.

The sections were cleaned using Phosphate-Buffered Water (PBS), followed by the addition of 1% Bovine Serum Albumin (BSA) to minimize nonspecific binding. The sections were rinsed with PBS and then exposed to the primary monoclonal antibody solution (iNOS, eNOS, nNOS 1:100 Santa Cruz Biotechnology) in a humid environment for 1 hour. Following a wash with Phosphate-Buffered Saline (PBS), the sections were subjected to a 20 minutes incubation period before the addition of the secondary biotinylated antibody. Following 20 minutes of stirring, PBS was employed to cleanse the streptavidin horseradish peroxidase enzyme mixture. The utilization of Diaminobenzidine (DAB) facilitated the detection of the initiation of an immunohistochemical reaction through its visible color change. The process was completed after the introduction of flowing water. Following a 30 second application of hematoxylin as a counterstain, the specimens were subsequently dried and mounted using mounting fluid. By utilizing the outcomes obtained from Adobe Photoshop version 7.2, we examined the appearance of NOS variations. Antibody-stained section micrographs were analyzed using pixels. The pixels revealed the presence of the biomarker (brown) and residual tissue (blue). To determine the expression ratio, we calculated the product of the total number of pixels (including both blue and brown pixels) and the number of pixels that correspond to the color of the biomarker. The data analysis was conducted using the Statistical Analysis in Social Science (SPSS) version 21.0 software. An independent t-test was employed to determine the group means. A p-value below 0.05 indicates the statistical significance of the difference. The results were utilized to determine the average \pm deviation of NOS isoforms for each group. We have previously published this methodology in several studies [23-29].

RESULTS

The expression of NOS isoforms in the cardiac tissue

As shown in table 1 and figure 1, the expression rate of iNOS in the heart tissue of control was 0.058 ± 0.017 and this was significantly increased in the heart tissue of the smoking group by 0.0925 ± 0.054 ($p=0.003$). The data showed that the expression rate of eNOS in the heart tissue of the control group was 0.129 ± 0.018 and this was significantly decreased in the heart tissue of the smoking group by 0.0725 ± 0.029 ($p=0.01$). The data regarding the expression rate of nNOS showed that it was in the heart tissue of the control group 0.058 ± 0.028 , while it was in the heart tissue of the smoking group 0.0752 ± 0.015 . This was not statistically significant ($p=0.058$).

Variable	Mean ± SD	p- value (Significance)
Smoking-iNOS	0.0925 ± 0.054	0.003
Control-iNOS	0.058 ± 0.017	
Smoking-eNOS	0.0725 ± 0.029	0.01
Control-eNOS	0.129 ± 0.018	
Smoking-nNOS	0.0752 ± 0.015	0.058
Control-nNOS	0.058 ± 0.028	

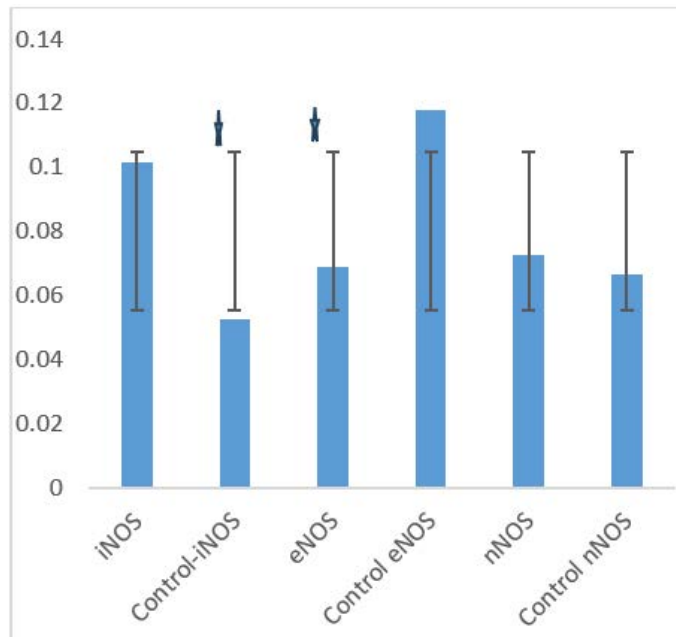


Fig. 1. The expression of NOS isoforms and their statistical significance

Immunohistochemistry of NOS isoforms in the cardiac tissues of control and smoking groups

As seen in figure 2, the expression of iNOS in the heart tissue of the control group was indicated. Antigenic sites representing the immunoreactivity of iNOS are indicated by the red arrow. Figure 3 represents the expression of iNOS in the heart tissue of smoking

rats.

Figures 4 and 5 represent the expression of eNOS in the control group and smoking group. The immunoreactivity of eNOS is represented by blue arrows.

The expression of nNOS is presented in figures 6 and 7 in the control and smoking group. The immunoreactivity of nNOS is denoted by the blue arrows.

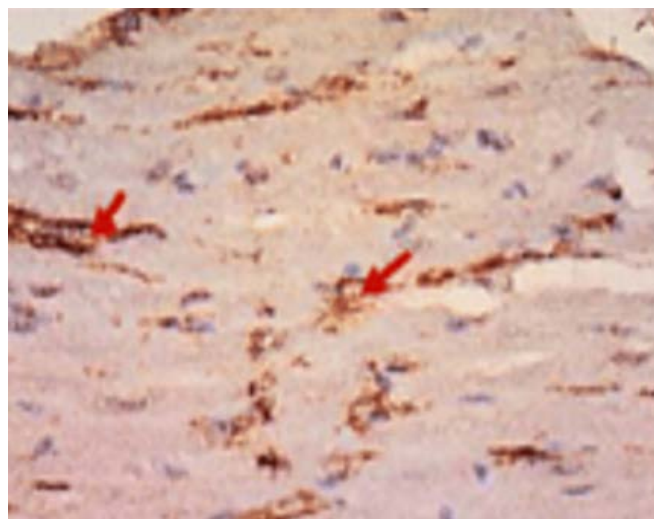


Fig. 2. The expression of iNOS in the heart tissue of the control group. Red arrows indicate the reaction sites (magnification 400X)

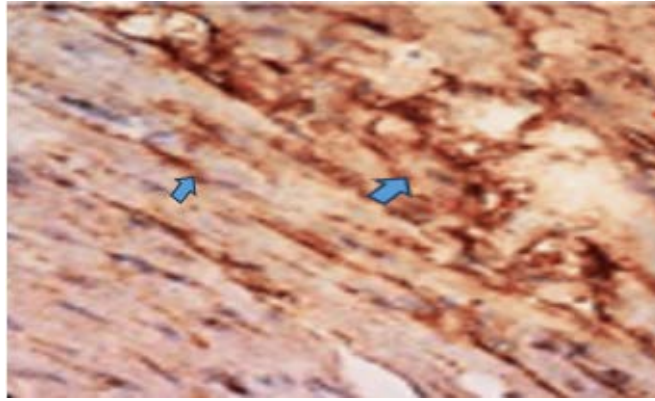


Fig. 3. The expression of iNOS in the heart tissue of the smoking group, blue arrows indicate the reaction sites (magnification 400X)

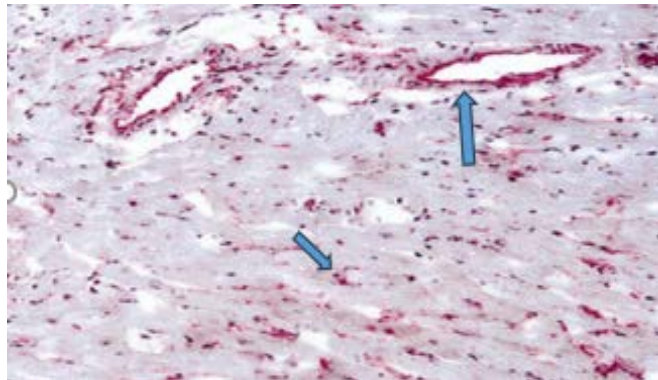


Fig. 4. The expression of eNOS in the heart tissue of the control group (magnification 40X)

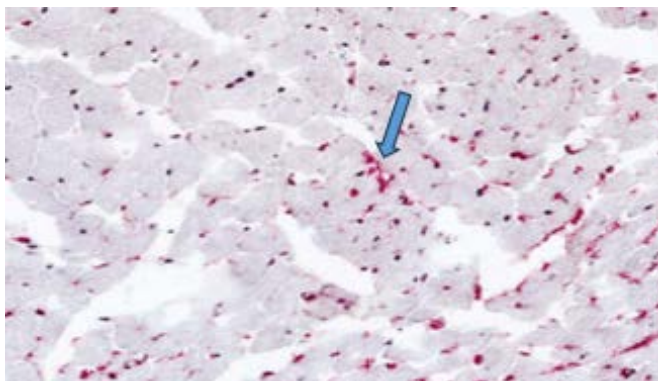


Fig. 5. The expression of eNOS in the heart tissue of the smoking group (magnification 40X)



Fig. 6. The expression of nNOS in the heart tissue of the control group (magnification 40X)

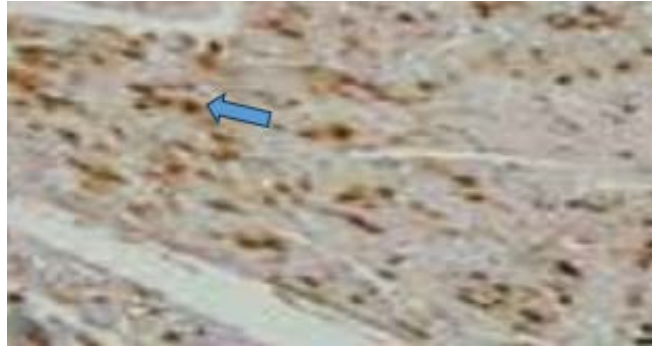


Fig. 7. The expression of nNOS in the heart tissue of the smoking group (magnification 40X)

DISCUSSION

The present study was conducted to investigate the expression of NOS isoforms in the heart tissue of smoking expression of iNOS. Previous studies showed that the expression of iNOS was increased in the heart in case of heart failure [30]. Smoking rodents may experience a substantial decline in cardiovascular health due to the elevated expression of Inducible Nitric Oxide Synthase (iNOS) in their cardiac tissues. Research publications support the subsequent potential effects. iNOS generation of NO worsens myocardial relaxation and contractility due to inflammation and oxidative stress [31]. Other impacts include myocardial infarction [32].

The results of the present study showed that the level of eNOS was significantly decreased in the heart of smoking rats compared with the control group ($p=0.01$). As for heart health, smoking rats showed considerably lower cardiac Endothelial Nitric Oxide Synthase (eNOS) levels than controls. This finding can be discussed given different considerations. Endothelial cells need eNOS to produce NO to blood pressure, platelet aggregation, and vascular

tone [33]. The deficiency of eNOS may lead to vasoconstriction and hypertension [34].

The results of the present study did not show a significant relationship between study groups in the concentration of nNOS ($p=0.058$). This finding can be discussed in the following context. Since there was no difference in nNOS expression across groups, smoking did not affect neuronal nitric oxide synthesis. This may imply that healthy and diseased NOS isoforms are regulated differently [34, 35].

CONCLUSION

Smoking negatively impacts heart health by promoting the up-regulation of iNOS expression and the downregulation of eNOS expression. This could account for various adverse health consequences linked to smoking that are related to the heart. The absence of a substantial correlation between the nNOS expression levels of the research groups suggests that the brain has a limited impact on heart disease.

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