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RESEARCH ARTICLE

Effect of *Nigella sativa extract* on nasal mucosal inflammation in rats exposed to cigarette smoke

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ABSTRACT

Cigarette smoke contains toxic compounds that can initiate an inflammatory reaction and infiltrate inflammatory cells. The nasal mucosa is the initial part of the respiratory system that comes into contact with irritating chemicals such as cigarettes. Prior studies indicated that *Nigella sativa* extract (NS) has thymoquinone, a bioactive compound with anti-inflammatory properties. Our study aims to determine the effect of different doses of NS on inflammation of the nasal mucosa in Wistar rats exposed to cigarette smoke. The study employed an experimental post-test-only control group design. Twenty-five Wistar rats were randomly assigned to one of the five groups: negative control (K-), positive control (K+), and three treatment groups of P1, P2, and P3 receiving NS at the dose of 0.5, 1, and 1.5 g/kg bw/day, respectively. All treatment groups and the positive control group were exposed to smoke from 4 cigarettes/day for 28 days. The degree of inflammatory cell infiltration was evaluated using a histological scoring : 0 if normal; 1 if infiltration was less than 1/3; 2 if infiltration was between 1/2 and 2/3, 3 if infiltration of more than 2/3 indicating normal, less than 1/3, 1/3 to 2/3, and more than 2/3, respectively. The degree of inflammatory cell infiltration in group: K(-): 1.00 ±0.00, K(+): 1.00±0.00, P1: 1.00±0.00, P2: 1.00±0.37, and P3: 1.00±0.33. The Kruskal-Wallis test showed a *p*-value of 0.001, indicating a significant difference. The Mann-Whitney test revealed significant differences between the K+ and P2 groups (p=0.032), K+ and P3 groups (p=0.013), and P1 and P3 groups (p=0.049). In conclusion, NS can decrease the presence of inflammatory cells in the nasal mucosa in Wistar rats exposed to cigarette smoke.

1. Introduction

Inflammation is a natural defense mechanism in response to tissue damage caused by physical, chemical, or microbiological factors. It serves as the immune system's response to harmful stimuli such as microorganisms and irritants and promotes tissue repair. The inflammatory response involves activating immune cells at the site of inflammation (Abdulkhaleq *et al.,* 2018; Andriyono, 2019). The microvascular inflammation leads to an increase in the permeability of capillary blood vessels. This increased permeability allows for the movement of leukocytes to the specific site of inflammation (Utami *et al.,* 2011). One of the causes of inflammation in the respiratory tract is exposure to cigarette smoke. Cigarette smoke can increase the production of proinflammatory cytokines, such as tumor necrosis factor-alpha (TNFα), interleukin-1 (IL-1), IL-6, IL-8, and granulocyte-macrophage colony-stimulating factor (GM-CSF) and increase the

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accumulation of immune cells in the airways. Thus, smoking can cause inflammation (Strzelak *et al.,* 2018). According to the Central Bureau of Statistics (BPS), the prevalence of smoking among children under age 15 in Central Java in 2020 was about 27.70% (Badan Pusat Statistik, 2020). The national household survey reported that in 2011, male smoke in Indonesia was around 67%, and passive smokers were around 78.4% (Global Adult Tobacco Survey (GATS), 2011).

Cigarette smoke is divided into mainstream and side-stream smoke. The former refers to smoke inhaled by the smoker 'slung through the burning cigarettes. The latter is known as side stream smoke, which refers to the smoke inhaled by a smoker and exhaled into the environment. Passive smokers are more adversely affected by cigarette smoke than active smokers. Studies demonstrated that sidestream smoke contains a more significant amount of tobacco combustion byproducts compared to mainstream smoke (Talumewo, 2012).

The nose is an integral part of the respiratory system. It effectively removes dirt and allergies from the air. The nasal epithelial cells are the initial entry portal of toxins in the inhaled air and serve as the primary barrier to infection (Jaspers, 2014). The gases and particles from cigarette smoke initially meet the immune system on the mucosal surfaces of the mouth cavity, sinuses, and airways. The combustion of cigarettes generates a multitude of reactive oxygen species (ROS). Cigarette butts do not effectively filter pollutants. Cigarette smoke ingredients, particularly ROS, stimulate intracellular signaling cascades in epithelial cells, activating inflammatory genes such as IL-8 and TNFα. The release of these inflammatory mediators enhances the attraction of inflammatory cells (Lee *et al.,* 2012). Cigarette smoke exposure can stimulate and generate innate and inflammatory immune cells, such as neutrophils and macrophages, leading to elevated levels of proinflammatory cytokines or chemokines, such as IL-8 and TNFα (Jasper *et al.,* 2014).

Acute and chronic inflammation treatments include non-steroidal anti-inflammatory drugs (NSAIDs), pain relief, corticosteroids, immune-suppressing drugs, and herbal supplements such as black cumin seeds. Black cumin seeds (*Nigella sativa* L.) have various pharmacological effects such as analgesic, antipyretic, anti-inflammatory, antinociceptive, antioxidant, antitumor and cytotoxic, anti-diabetic, and anti-ulcer effects (Yoruk *et al.,* 2017). Black cumin seed has been shown to inhibit proinflammatory cytokines and TNFα. It has been shown to have anti-inflammatory activity, inhibiting the synthesis of inflammatory cytokines, namely leukotrienes, and thromboxanes, by inhibiting the activity of lipoxygenase (LOX) and cyclooxygenase (COX) (Khan *et al.,* 2016). Thymoquinone, one of the active substances in N, can suppress inflammatory reactions caused by mast cells by modulating the proinflammatory transcription factor NF-Kβ and preventing transcription and TNFα production (Amanulloh & Krisdayanti, 2019). This study aims to examine the effects of multi-dose NS extract on the degree of inflammatory cell infiltration in the nasal mucosal inflammation in Wistar rats exposed to cigarette smoke

2. Materials and Methods

This was a post-test-only control group study in Wistar rats. The research was conducted at the Animal Experimental House, The Faculty of Mathematics and Natural Sciences, Semarang State University; the Experimental Animal Laboratory, Medical Faculty, Diponegoro University; and the Anatomical Pathology Laboratory at the Diponegoro National Hospital (RSND). The research samples were male Wistar rats aged 8-12 weeks weighing 200-250 g. The sick/unhealthy, inactive, and anatomically abnormal rats were excluded, especially in the nose and ears. The rats that died during the research and before being sampled for observation were not included in this research.

A total of 25 Wistar rats were randomly divided into five groups consisting of two control groups and three treated groups. The negative control (K-) group was only given a standard diet, and the positive control (K+) group was exposed to cigarette smoke. The Treatment 1 (P1), treatment 2, and Treatment 3 groups were given 0.5 g/kg bw/ day *Nigella sativa extract* (NS); treatment 2 (P2) group was given NS at dose 1 g/kg BW/day; treatment 3 (P3) group which was given NS at dose 2 g/kg bw/day. All treatment groups were exposed to the smoke of 4 cigarettes/day for 30-minute intervals of the administration of NS. The length of the experimental animal treatment was 28 days. The rats were terminated on day 29 using chloroform inhalation and were assessed for inflammatory cell infiltration by hematoxylin-eosin (HE) staining. The readout used 400 x magnifications in five fields of view. The degree of inflammatory cell infiltration was classified into a score of 0 (no histopathological changes), 1 (inflammatory cell infiltration <1/3 field of view), 2 (inflammatory cell infiltration of 1/3-2/3 field of view), and 3 (inflammatory cell infiltration >2/3 field of view).

The data were statistically analyzed using the Kruskal-Wallis test to determine the difference between the two treatment groups, followed by the Mann-Whitney U test. The ethical clearance of this study was obtained from the Health Research Ethics Commission (KEPK), Medical Faculty, Diponegoro

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University; Semarang (No. 111/EC/H/FK-UNDIP/IX/2021).

3. Results

The nasal mucosal respiratory preparations of all groups at 400x magnification are shown in Figure 1. Table 1 shows the mean inflammatory cell infiltration after treatment. Besides, it shows that the degree of inflammatory cell infiltration is inversely proportional to the dose of NS seed extract, the higher the dose, the more significant the decrease in the degree of inflammatory cell infiltration. Kruskal-Wallis test

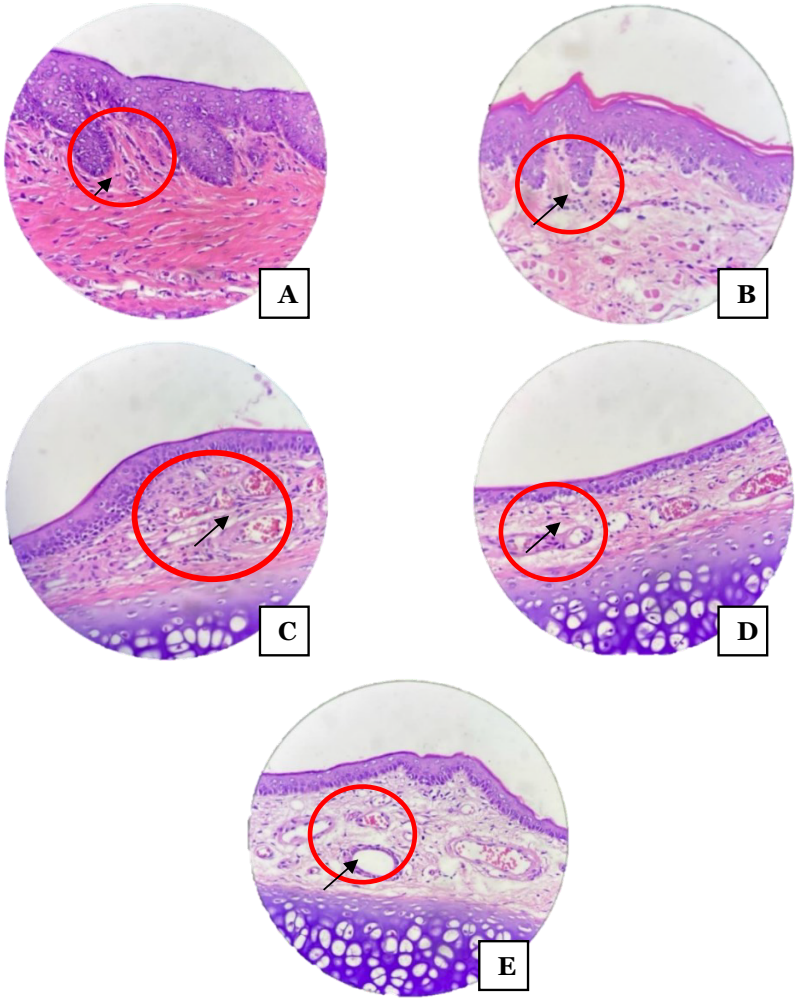

Figure 1. Microscopic observations of inflammatory cell infiltration in the lamina propria. (A) Inflammatory cell infiltration <1/3 LP group K-, (B) inflammatory cell infiltration 1/3-2/3 LP group K+, (C) Cell infiltration inflammation 1/3-2/3 LP group P1, (D) Inflammatory cell infiltration <1/3 group P2, (E) Inflammatory cell infiltration <1/3 LP group P3. LP = field of view. = inflammatory cell infiltration. = inflammatory cells.

Table 1. Comparison of the degree of inflammatory cell infiltration in all groups

Groups	Inflammatory Cell Infiltration				
	0	1	2	3	Median
Negative control (K-)	0	25	0	0	1.00 ±0.00[a]
Positive control (K+)	0	14	11	0	1.00±0.00[b]
0,5 g/kg bw/day of NS (P1)	0	16	9	0	1.00±0.00[c]
1 g/kg bw/day of NS (P2)	0	21	4	0	1.00±0.37[d]
1.5 g/kg bw/day of NS (P3)	0	22	3	0	1.00±0.33[a]

Note: the different superscript showed a different significance ($p<0.01$) compared to the negative control based on Mann-Whitney U test

showed a significant difference (p = 0.001) in the degree of inflammatory cell infiltration of the all groups.

4. Discussion

Rats were assumed to be passive smokers exposed to cigarette smoke. Through exposure to cigarette smoke, the flow of toxic substances was inhaled through the nasal. The nasal mucosal epithelium was the primary site of infection (Jaspers, 2014). Cigarette smoke gases and particles first interact with the immune system on the mucosal surfaces of the oral cavity, sinuses, and airways (Lee *et al.,* 2012). Inhalation of cigarette smoke could activate endogenous mechanisms, including the accumulation of neutrophils and macrophages that can potentially increase pulmonary damage (Zahara, 2019). The concentration of Reactive Oxygen Species (ROS) in cigarettes could cause the inflammatory mediators' activation (e.g., IL-8 and TNFα). The secretion of these mediators could lead to increased recruitment of inflammatory cells (Zahara, 2019). However, in this study, the proinflammatory cytokine factors were not assessed.

In the control group, inflammatory cell infiltration was obtained with a score of 1 in the field of view. Cell damage and inflammation, as indicated by the infiltration of inflammatory cells, namely leukocytes, especially neutrophils, arose as a normal endogenous response to free radicals resulting from internal and external biochemical chain processes in the body (Fauzi, 2018; Ganesha *et al.,* 2020). Thus, inflammatory cell infiltration could occur under normal conditions. A previous study of histopathology inferior turbinate in hypertrophy turbinate patients showed similar results: the most common goblet cell hyperplasia found was grade 3 (48%), the most submucosal gland formation was grade 2 (51%), and the most inflammatory cell infiltration in lymphocytes was grade 2 (21%) (Dewi *et al.,* 2020).

Based on the research results in the K+ group, there was an increase in inflammatory cell infiltration with a score of 2 and no infiltration with a score of 3. In the previous research, exposure to 5 cigarettes in 1 h/day and 5 days/week, on day 14, neutrophils were rarely found, but there was an increase of mast and eosinophil cells. Meanwhile, on day 28, a chronic picture of the epithelium and the lamina propria was viewed. In the lamina propria, there were histological changes, namely glandular hypertrophy, fibroplasia of the connective tissue, and increased infiltration of eosinophils, lymphocytes, and a few neutrophils (Trombitas *et al.,* 2016). The wound healing phase comprised hemostasis, inflammation, proliferation, and remodeling. Leukocyte infiltration could be observed within 24 to two days, while neutrophils began appearing only a few hours after the lesion. The inflammatory phase started after the lesion and lasted 4-6 days. The proliferative phase consisted of re-epithelialization, angiogenesis, granulation tissue formation, collagen deposition, and fibroplasia. This phase occurred the 2nd to the 14th day after the lesion. The remodeling phase could begin 2-3 weeks after the lesion and last for a year or more (Gonzalez *et al.,* 2016; Politis *et al.,* 2016; Selvarajah *et al.,* 2020).

Based on the Mann-Whitney U test, there were significant differences between the inflammatory cell infiltration of the K+ group with P2 and P3 groups and the inflammatory cell infiltration of the P1 and P3 groups. In the intervention group, the most significant difference was found between the infiltration of inflammatory cells in the K+ and P3 groups. It proved the theory that *Nigella sativa* could act as an anti-inflammatory. (Yoruk *et al.,* 2017). Thymoquinone is one of the active substances in *Nigella sativa*, which could reduce cytokines produced by Th2, including IL-4, IL-5, and IL-13, and decrease the amount of serum IgE. The inflammatory response and mucosal edema could be prevented by reducing IL-4 and serum IgE levels (Amanulloh & Krisdayanti, 2019; Rahmawati *et al.,* 2021).

In the P1 group, there were no significant differences from the K+ group. It might be due to a low dose. The inflammatory process and cell infiltration due to toxic substances or free radicals from cigarette smoke could not be inhibited at 0.5 g/kg bw/day. It was in line with previous research regarding the qualitative assessment of the histology of the heart; there was still much congestion, and there were inflammatory cells in the form of neutrophils and lymphocytes on microscopic examination with *Nigella sativa extract* a dose of 0.6 g/kg bw/day (Wijaya, 2017)

There was a significant difference between treatment groups and control groups. The P2 group differed significantly from the K- and K+ groups. It indicated that NS at the dose of 1 g/Kg BW/day decreased the degree of inflammatory cell infiltration but was not close to the normal group. At the same time, the P3 group had a significant difference with the K+ group but had a non-significant difference with the K- group. It explained that an NS dose of 2 g/kg bw/day reduced the degree of inflammatory cell infiltration, with results approaching the normal group.

Thymoquinone had the role of inhibiting the synthesis of inflammatory mediators, namely leukotriene B4 and thromboxane B2, by inhibiting the activity of lipoxygenase (LOX) and cyclooxygenase (COX) in arachidonic acid metabolism. Inhibiting inflammatory mediator synthesis could prevent further inflammation and cell damage (Zahara, 2019). In

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addition to inhibiting inflammatory mediators through the arachidonic acid metabolic pathway, thymoquinone could reduce ROS from cigarette smoke so that the damage caused was minimal (Zahara, 2019). *Nigella sativa* could inhibit proinflammatory cytokines and TNFα (Khan *et al.,* 2016). Inflammatory mediators, such as IL-7 and TNFα, could increase inflammatory cell recruitment. (Zahara, 2019)

Nigella sativa seed at a dose of 2 g/kg bw/day showed a significant change compared to the K+ group, but there were still inflammatory cells. This research was in line with previous research. Research on the effect of thymoquinone on the rats' pulmonary showed that there was a significant decrease ($p<0.005$) of neutrophils in the alveolar space, neutrophils in the interstitial space, and thickening of the alveolar septum in the rats' exposed to cigarette smoke and given thymoquinone (Zahara, 2019), Thymoquinone could inhibit the production of IL-4, OVA-specific IgE, expression of TNFα, and IL-1β on rats with allergic rhinitis. Thymoquinone decreased eosinophil infiltration and nasal mucosal edema but did not affect increasing IFN-γ and IL-10 (Günel *et al.,* 2017). *Nigella sativa extract* at a dose of 2.4 g/kg bw/day showed no congestion and reduced inflammatory cell infiltration (Wijaya, 2017).

5. Conclusions

Nigella sativa seed extract could reduce the degree of inflammatory cell infiltration in the nasal mucosal of Wistar rats exposed to cigarette smoke.

Conflict of interest

All authors have no conflict of interest in this article.

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