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Original Research Article

The Protective Effect of N-Acetylcysteine and Honey Against Lungs Damage of Mice (*Mus Musculus*) After Cigarette Smoke Exposure: A Histological Study

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Abstract

Background: Cigarette smoke is a source of free radicals that could cause lung damage. Honey and N-Acetylcysteine have antioxidant effects that could play a role in preventing lung damage.

Objective: This study aims to compare the effects of honey and N-Acetylcysteine on lung histological features of mice (*Mus musculus*) after exposure to cigarette smoke.

Methods: This research is an actual experimental study with **post-test only control group design**. There were 25 adult male mice selected and divided into four groups, i.e., the standard control group (not given exposure or treatment); the negative control group (received 0.2 mL of aqua bidest and exposure to cigarette smoke); the first treatment group (received 0.2 ml of honey and exposure to cigarette smoke); the second treatment group (received 0.2 mL of N-Acetylcysteine and exposure to cigarette smoke). The treatment was carried out every day for 21 days.

Result: One-way ANOVA statistical test showed significant differences between the experimental groups ($p<0.001$). Mice in the group that was only received aqua bidest (negative control group) had the highest percentage of lung damage (76.6%) compared to mice from other groups. The lowest percentage of lung damage was found in the treatment group that received NAC (26.6%). Tukey test showed no significant differences in the lung damage between honey and N-Acetylcysteine groups ($p=0.685$).

Conclusion: Honey and N-Acetylcysteine demonstrated some protective effect against lung damage of mice as a result of cigarette smoking exposure. Further research is recommended by extending the treatment time, increasing the exposure to cigarettes smokes per day, as well as varying the dose of honey and N-Acetylcysteine administered.

Keywords: *Cigarette smoke, Honey, Lung Histology, Mice (Mus musculus), N-Acetylcysteine*

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INTRODUCTION

Smoking is a great public health issue all over the world and an important cause of morbidity and mortality.[1] The tobacco epidemic is one of the biggest public health threats the world has ever faced, killing more than 8 million people a year, including around 1.2 million deaths from exposure to second-hand smoke.[2] Tobacco smoke contains a mixture of chemicals, including a host of reactive oxygen- and nitrogen species (ROS and RNS), among others, that can damage cellular and sub-cellular targets, such as lipids, proteins, and nucleic acids. A growing body of evidence supports a key role for smoking-induced ROS and the resulting oxidative stress in inflammation and carcinogenesis.

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There is ample evidence to support that habitual smoking is a primary risk factor for chronic obstructive pulmonary disease (COPD), cardiovascular disease (CVD), immune-mediated inflammatory diseases, and a variety of cancer types.[3]

A research found that to prevent, slow, and improve the negative impact of free radicals (electron acceptor) derived from cigarette smoke on health, antioxidant (electron donor) is required.[4] An antioxidant is a substance or molecule that could slow or prevent oxidation damage from other molecules including free radicals. N-acetylcysteine (NAC) is a thiol compound that was developed as is a mucolytic agent as it breaks down mucin disulfide cross-links to reduce mucus viscosity. It also has antioxidant properties through increasing glutathione concentrations, which are reduced in COPD.[5]

Antioxidants could derive from drugs or natural sources. Honey is a nutritional antioxidant that is naturally processed by honey bees.[6] Honey has a potential therapeutic role in the treatment of disease by phytochemical, anti-inflammatory, antimicrobial, and antioxidant properties. Flavonoids and polyphenols, which act as antioxidants, are two main bioactive molecules present in honey.[7]

Since the exposure to cigarette smoke will result into free radicals, it is therefore necessary to take a preventive measure such as antioxidants both through drugs and from natural sources. This study aims to compare the effects of the administration of honey and the drug N-Acetylcysteine on the histological image of lung mice (*Mus musculus*) after exposure to cigarette smoke.

MATERIALS AND METHODS

Research Design

This was a true experimental study with **post-test only control group design**, where the main group was randomly divided into two main groups: the control and the treatment group.[8] After intervention was given to the treatment group, assessment was carried out in intervention and control group.

Research Sample

Twenty-five mice (*Mus musculus*) adult males (Federer formula) weighing ± 20-30 gr were included in our analysis.[9] Samples were selected randomly and grouped into four groups:

1. Standard control group: not administered exposure to cigarette smoke, honey solution, or N-Acetylcysteine.
2. Negative control group: administered aqua bidest and exposure to cigarette smoke.
3. First treatment group: administered a honey solution and exposure to cigarette smoke.
4. Second treatment group: administered N-Acetylcysteine and exposure to cigarette smoke.

Research Procedure

Before starting the study, all mice matured for seven days. The purpose of this acclimatization was to evaluate if all the mice could adapt to the conditions during the experiment. During the acclimatization process, mice were given foods and drinks moderately every day.

Administration of honey

The honey product used was HDI Naturals™ Clover Honey. The honey solution was administered ± 30 minutes before exposure to kretek cigarette smoke for 21 consecutive days. The dose of honey solution administered was 0.2 mL/mouse in the first treatment group. The honey solution was administered using a syringe and an oral gastric tube.

Administration of N-Acetylcysteine

The drug solution was administered ± 30 minutes before exposure to cigarette smoke for 21 consecutive days. The dose of drug solution administered was 0.2 mL/mouse in the second treatment group. The drug solution was administered using a syringe and an oral gastric tube.

Exposure to cigarette smoke

The *kretek* cigarette smoke was administered to each trial group (except the standard control group) after ± 30 minutes of honey and N-Acetylcysteine for 21 consecutive days at a dose of one cigarette per head each day. Exposure was performed by inserting cigarettes into a smoking pump and then burned them; then. The smoke was channeled into a smoking chamber measuring 60 cm x 30 cm x 25 cm.

Observation of histological images of the lungs

After exposure to cigarette smoke for 21 days, surgery was carried out, followed by lung preparations using Hematoxylin-Eosin (HE) staining. Observations was made using a light microscope based on the field of view with a magnification of 40 times, 100 times, and 400 times. The parameters assessed were dilation of the lumen alveoli and infiltration of inflammatory cells with the following criteria:[10]

Scoring dilation lumen alveoli:
Score 0: No histological changes
Score 1: damage to less than 1/3 of the field
Score 2: there is damage at 1/3 to 2/3 roomy view
Score 3: there is damage to more than 2/3 of the field
Scoring infiltration of inflammatory cell:
Score 0: No histological changes
Score 1: infiltration of inflammatory cells at less than 1/3 of the field of view
Score 2: infiltration of inflammatory cell damage at 1/3 to 2/3 roomy view
Score 3: infiltration of inflammatory cells in more than 2/3 roomy view

The total values of the two parameters were summed and divided by six (maximum number of scores) and then multiplied by 100% to calculate the degree of lung damage as s shown in Table 1.[10]

Table 1. Determination of the extent of lung damage

Percentage of damage (%)	Degrees of damage
0	No damage
< 30	Mild damage
30 – 60	Moderate damage
> 60	Severe damage

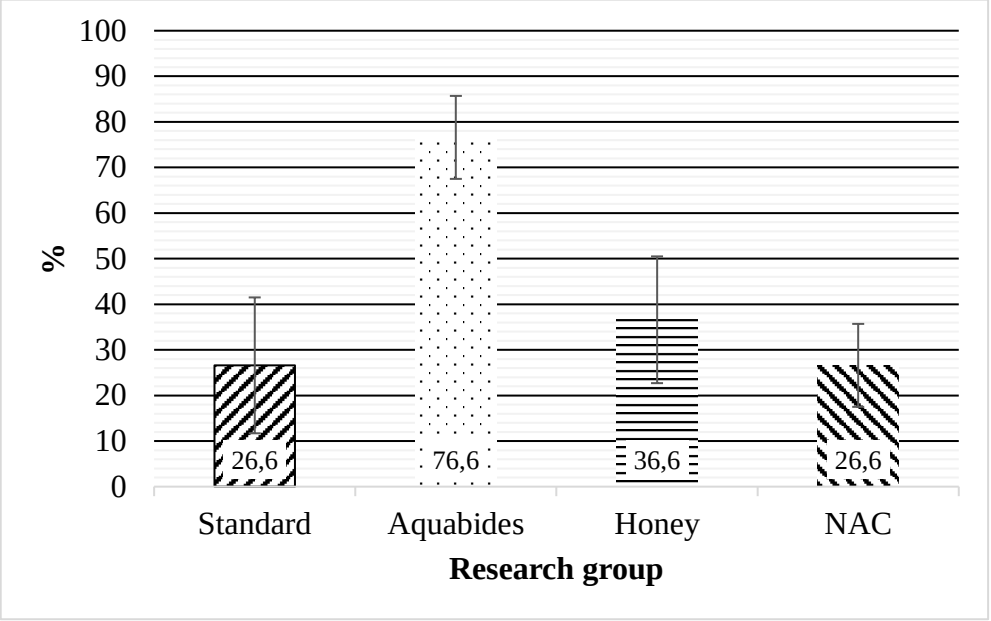

Figure 1. Graph of the average percentage of lung damage after exposure to cigarette smoke for 21 days. (NAC = N-Acetylcysteine)

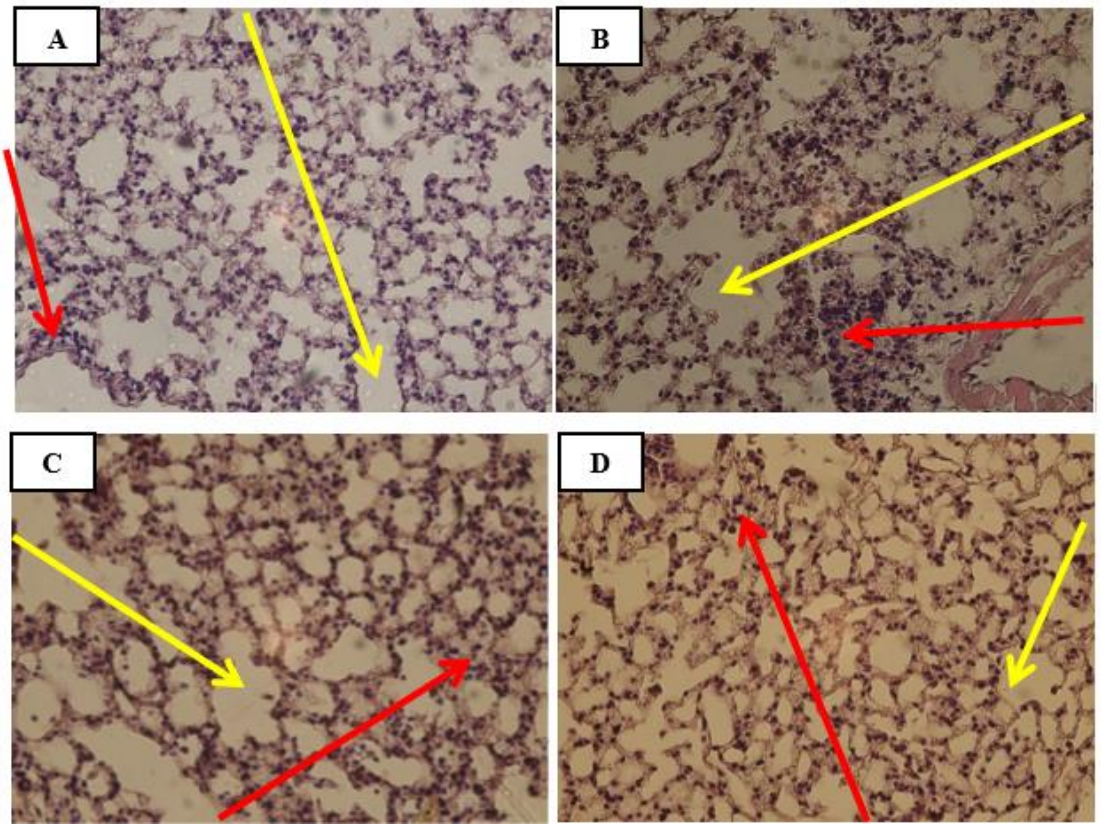

Figure. 2. Histological description of lung damage parameters. A. Not exposure or treatment; B. Cigarette smoke + aquabidest; C. Cigarette smoke + honey; D. Cigarette smoke + N-Acetylcysteine. Description: Arrow yellow (dilation of lumen alveolus); Arrow red (infiltration of inflammatory cells). Hematoxylin-Eosin (HE) staining . 400 times magnification

Data Analysis

Data analysis was performed using one-way ANOVA statistical test. Previously, normality test (Kolmogorov-Smirnov test) and data homogeneity test (Levene tests) were carried out, as the condition for ANOVA one-way test. When the results of the ANOVA one-way test were significant ($p<0.05$), a post hoc analysis was conducted using the Tukey test to find out which group pairs had significant differences across groups.[11]

RESULTS

Exposure to 21 days of cigarette smoke in each group showed damage to lung tissue of mice (Figure 1). Figure 1 shows that the group that was only given aqua bidest (negative control group) was unprotected from lung damage due to cigarette smoke exposure. This was

reflected by its high percentage of lung damage (76.6%) in this group. The lowest percentage of lung damage was found in the second treatment group that received NAC (26.6%), which was also very similar to the percentage of lung damage in mice from the standard control group (not exposed to cigarette smoke and did not receive any treatment).

Figure 2 shows the histological image of the lungs in each experimental group. The aqua bidest group, showed an increased widening of the alveolus lumen (Figure 2.B1) and increased inflammatory cell infiltration (Figure 2.B2), compared to the standard control group. In the groups of which honey or N-Acetylcysteine was administered (Fig 2.C,D), there was a decrease in the lumen of alveolus that experienced dilation and infiltration of inflammatory cells, compared to the aqua bidest group (negative control group). The statistical analysis showed that there was no difference in the effect of administering honey and N-Acetylcysteine on the histology image of lung mice due to exposure to cigarette smoke (p=0.*685*)

DISCUSSION

Lung damage of mice due to cigarette smoke exposure

Our finding showed that cigarette smoke exposure could cause lung damage in mice. This was shown by mice which were exposed to cigarette smoke and only received aqua bidest. Mice in this group demonstrated the most severe damage of the lungs compared to mice from other groups. Free radicals derived from cigarette smoke could cause lung damage through the mechanism of oxidative stress.

Cigarette smoke causes oxidative stress resulting in a chronic low-grade inflammation and recruitment of inflammatory cells to the airways by activation of epithelial cells, AMs, neutrophils and T lymphocytes. Furthermore, cigarette smoke increases the virulence of pathogens, increasing the risk of pulmonary infections.[12] Cigarette smoke exposure induces the autophagy process to maintain cellular homeostasis. Additionally, autophagy flux may contribute to increasing epithelial cell loss. Autophagy components including autophagy-related protein (ATG) family kinase, Unc-51-like autophagy activating kinase (ULK1), and ULK2 in mammals are key regulators of autophagy initiation. AMP-activated protein kinase (AMPK) is another strong candidate for influencing autophagy function and acts as an energy sensor to regulate cellular homeostasis. AMPK induces autophagy in response to different cellular stresses, including oxidative stress. In response to irritant exposure, reactive oxygen species (ROS) activation, inflammatory response, and apoptosis occur, damaging the lung alveoli and leading to pulmonary pathologies, including chronic obstructive pulmonary disease (COPD)-emphysema.[13]

The parameters of the degree of lung damage to the dilation of the lumen alveolus indicated the presence of emphysema induced by damage to lung elasticity in the respiratory process. Emphysema is defined as persistent abnormal widening of the air spaces (alveoli distal to the terminal bronchioles) with destruction of their walls without marked fibrosis. The pathogenesis f emphysema involves several mechanism and hypotheses regarding proteases and antiproteases and is a major concern because according to the scenario the release of proteases by cigarette smoke exposure that inhibits the antiprotease response results in degradation of the extracellular matrix (elastin and collagen).[14]

Alveolar macrophages play a central role in airway inflammation. These cells secrete multiple chemokines and cytokines, such as tumor necrosis factor-α (TNF-α), that induce the expression of adhesion molecules on endothelial cells, facilitating the migration of a variety of inflammatory cells. Alveolar macrophages also produce ROS, metalloproteinases (MMPs), and cathepsins to disrupt alveolar structures and induce fibrosis mediators such as TGF-β1 to trigger airway remodeling. Neutrophils are an important component of type 1 inflammation. Neutrophil migration to the lungs is caused by the accumulation of ROS induced phosphatidylinositol 3,4,5-triphosphate at the injury site. Airway neutrophils secrete myeloperoxidase, neutrophil elastase (NE), cathepsins, proteinase, and MMPs and are directly involved in the destruction of alveoli and promote mucus production in the submucosal glands and goblet cells.[15]

The effect of administering honey on lung damage of mice after exposure to cigarette smoke

We found that mice which were exposed to cigarette smoke and received honey suffered from only moderate lung damage. This condition was shown by the decrease widening of the lumen of the alveolus and decrease infiltration of inflammatory cells. There was a significant difference between the percentage of lung damage between mice in this group that to mice in the aqua bidest group.

Honey serves as a source of natural antioxidants, which play an important role in human health by combating damage caused by oxidizing agents.[16] This study shows that honey could prevent cell damages in the lung organs since it contains mineral nutrients and antioxidant compounds. Honeybee products are considered to be a rich source of antioxidants compounds (especially phenolic acid and favonoids) and, therefore, they have a significant place among natural products that can scavenge free radicals and counteract oxidative stress-induced cellular damage.[17] The neutralization of free radicals by antioxidant molecules can occur either by directly reacting with them or they may become less active free radicals and less dangerous than those they have neutralized, though dietary intake of antioxidants maintains satisfactory antioxidant status in the body. Flavonoids present in honey have been demonstrated as very effective scavengers of reactive oxygen (ROS) and reactive nitrogen species (RNS) (like peroxyl, alkyl peroxide, hydroxyl, superoxide radicals, nitric oxide, and peroxynitrite) to counter the oxidative damage induced by these molecules. Flavonoid structure is attributed with three chemical features, presence of a 2, 3 double bond in the C-ring, B-ring having ortho-dihydroxy structure and presence of a 4-oxo function. On the B-ring, hydroxyl groups donate an electron and hydrogen to stabilize peroxynitrite and peroxyl and hydroxyl radicals making comparatively stable flavonoid radicals.[18]

The phenolic acids and flavonoids are responsible for the well-established antioxidant activity of honey. Apart

from these, sugars, proteins, amino acids, carotenes, organic acids, Maillard reaction products, production of reactive oxygen species (ROS), and other minor components also contribute to antioxidant effect. The antioxidant properties of honey can be measured in the form of antiradical activity using, oxygen radical absorbance capacity (ORAC) assay, 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging assay, and ferric reducing antioxidant power (FRAP) assay.[19]

The effect of N-acetylcysteine treatment on lung damage of mice after exposure to cigarette smoke

The results showed that mice exposed to cigarette smoke and received N-Acetylcysteine only showed mild damage rates. The mice in this group had the lowest average percentage of lung damage compared to mice from other groups. We also found a significant difference between the mice exposed to cigarette smoke and received N-Acetylcysteine than mice from the aqua bidest group. This result confirmed the antioxidant effect of N-Acetylcysteine in preventing lung damage as a result of cigarette smoke exposure.

N-acetylcysteine (NAC) is a mucolytic agent as it breaks down mucin disulfide cross-links to reduce mucus viscosity (5). NAC is a thiol that acts as an acetylated precursor to the amino acid L-cysteine; it can reduce various radicals, by donating one electron, or acts as a nucleophile by donating one or two electrons. Its chemical structure, formed by the sulfhydryl functional group (–SH) plus an acetyl group (–COCH3) linked to the amino group (NH2), is responsible for its metabolic activities related to the direct and indirect antioxidant action and mucolytic action.[20]

The direct antioxidant activity of NAC is due to the ability of its free thiol group to react with reactive oxygen and nitrogen species (RONS). Under experimental conditions, NAC reacts quickly with the hydroxyl radical (OH) nitrogen dioxide (NO2), carbon trioxide ion ($CO3^{-}$), and thiyl radical (RS), in addition to the nitroxyl (HNO) that is the reduced and protonated form of nitric oxide (•NO).[20] On the other hand, it is often assumed that the antioxidative agency of NAC can be explained by its ability to act as a source of cysteine (Cys) for increased glutathione (GSH) biosynthesis.[21] Glutathione is one of the antioxidants in the body. Glutathione will keep the DNA and RNA chains in the cell nucleus from breaking down and protecting the cell nucleus from free radicals.[22] Based on research conducted by Aldini et al. [23], N–Acetylcysteine's indirect antioxidant activity was a precursor to Glutathione (GSH). This condition indicated the ability of N-Acetylcysteine to increase back Glutathione mutation levels that decrease due to oxidative stress.

The pathogenesis and progression of respiratory diseases can be explained by inflammation and increased oxidative stress, with a consequent reduction in endogenous antioxidants, such as GSH. NAC represents a promising therapeutic target for the treatment of these diseases, including chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), and idiopathic pulmonary fibrosis (IPF), due to its antioxidant, anti-inflammatory, and mucolytic properties already described, related to its ability to replenish the GSH intracellular pool and reduce mucus production and viscosity.[20]

Effect of honey and N–Acetylcysteine on lung damage of mice after cigarette smoke exposure

The results showed no significant difference in the effect between the administration of honey and N-Acetylcysteine on the histological condition of the lungs after exposure to cigarette smoke. This shows that honey and N-Acetylcysteine have similar abilities to prevent lung damage as a result of cigarette smoke exposure. This might be due to the antioxidant effects of honey and N-Acetylcysteine which could neutralize free radicals from cigarette smoke exposure. However, in this study, N-Acetylcysteine showed a better effect than honey in preventing lung damage. This supported the finding from Eserina et al, that reported the antioxidant mechanism of N-acetylcysteine through chemical process.[21]

Strengths and limitations of the study

The advantage of this research is the use of honey products which have been proven to be processed naturally without the addition of other ingredients/ substances and have been standardized internationally. The limitation of this study was the limited number of cigarettes (one cigarette/day) used to provide cigarette smoke exposure to mice.

CONCLUSIONS

In summary, honey and N-Acetylcysteine showed their protective effect against mice lung damage after cigarette exposure. Although mice who received N-Acetylcysteine had the lowest percentage of lung damage compared to other groups, the percentage of lung damage between mice in this group and in honey group was not significantly different. Our findings provide important information for humans to consume substances that contain antioxidants to prevent lung damage due to free radicals, particularly from cigarette smoke exposure. It is also recommended to conduct further research by extending the treatment time, increasing the exposure to cigarettes smokes per day, and varying the dose of honey and N-Acetylcysteine administered. In addition, further research could be carried out on the accuracy of the N-Acetylcysteine dose that could be administered to patients with COPD to prevent further lung damage.

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