CLINICAL & BASIC RESEARCH

The Molecular Pathways of Lung Damage by E-Cigarettes in Male Wistar Rats

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ABSTRACT: *Objectives:* This study aimed to analyse the stages of lung tissue damage caused by exposure to electronic cigarette (e-cigarette) smoke. The negative health effects of e-cigarettes remain debatable. Several studies have shown the adverse effects, but others opine that e-cigarettes are safer to use than their tobacco counterparts. There is, however, a possibility that the long-term health effects, such as respiratory and even kidney function impairment, are presently not obvious. The amounts of increased free radicals and pro-inflammatory cytokines from e-cigarettes result in various physiological disorders, which trigger cell damage and even cell death in the body. *Methods:* An experimental study was conducted between March and September 2019 in Airlangga University using a control and an experimental group of male Wistar rats to assess the levels of malondialdehyde, interleukin (IL)-8, IL-10, matrix metalloprotein-8 and type-2 collagen. The results were obtained using immunohistochemical staining methods on alveolar macrophages through Hematoxylin-Eosin staining. *Results:* The results showed that exposure to e-cigarette smoke caused an increase in free radicals, triggered an inflammatory process and degraded the type-2 collagen present in the lung tissue. *Conclusion:* Exposure to e-cigarette smoke can cause cell damage in lung tissues.

Keywords: E-Cigarettes; Lungs; Immunohistochemistry; Hematoxylin; Eosin.

Advances in Knowledge
- *This study explains the mechanism of lung tissue damage due to exposure to electronic cigarette (e-cigarette) smoke from the airway to the cells of lung tissues.*
- *The damage caused by e-cigarettes reaches the type-2 collagen, which indirectly affects the cartilages of the respiratory tract.*

Application to Patient Care
- *This research aims to analyse the impact of the damage caused by e-cigarettes to enable its use as a reference in educating the public to avoid or reduce using them.*

THE USE OF ELECTRONIC CIGARETTES (e-cigarettes) increases every year. They are usually advertised as products that have low nicotine levels and are often seen as a viable alternative to smoking cessation. Consequently, most people believe e-cigarettes are safer than their tobacco counterparts.[1] In addition, aggressive marketing from e-cigarette producers accelerates and increases its use among users.[2] While various studies have shown the short-term adverse effects, such as an increase in free radicals and/or respiratory/airway inflammation, the long-term risks of exposure and use remain unknown because it is still a recent innovation.[3,4] However, various physiological disorders arising from cell damage caused by e-cigarette smoke pose a safety concern.[5]

The e-cigarette is an electronic device that utilises battery power.[6] Its use is different from tobacco cigarettes, which require the burning of natural tobacco leaves. It involves a heating process conducted on metals containing a liquid, either nicotine, propylene glycol or vegetable glycerol, which is used as a solvent or flavour.[6] The metal and the liquid are heated to give off steam that is inhaled by the user. The various types of metals and materials used in the manufacturing process of these e-cigarettes increasingly raise concerns regarding its level of safety for users.[7] The resulting steam produced also causes inflammation in the airways, reduces immunological defence and triggers lung tissue damage.[8]

E-Cigarettes contain high concentrations of free radicals that are approximately 1016 molecules/puff. These free radicals, in turn, consist of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Upon inhalation, free radicals are neutralised by enzymatic antioxidants such as superoxide dismutase, glutathione peroxidase and catalase. However, when these free radicals are inhaled or taken in excess, complete neutralisation will be impossible. This would consequently trigger cellular damage and inflammatory response through the release of pro-inflammatory cytokines by macrophage cells.[9] One of these cytokines is interleukin-8, which plays a role in cellular rupture and damage. Other cytokines include the neutrophil chemotactic factor, which releases neutrophils into lung tissue. Increased interleukin (IL)-8 secretion will stimulate the release of a metalloprotein-8 matrix into the lung tissues.[10]

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Metalloprotein matrix is an endopeptidase synthesised by neutrophils and expressed in various inflammatory cells when these neutrophils are activated by different inflammatory mediators of cell damage.[11] Therefore, metalloprotein matrix is not expressed in healthy cells or tissues, so it is often a parameter for the diagnosis of tissue damage.[12] Excessive metalloprotein-8 matrix will affect the collagen structure and function in respiratory cartilages by increasing the degradation of type-2 collagen.[13] Such damage occurring in the lung tissue for an extended amount of time would increase the risk of pulmonary fibrosis. This would render the tissue damage irreversible and result in various diseases, including pulmonary hypertension, cardiovascular problems and lung cancer.[14]

This study aims to determine the stages of lung tissue damage through increased free radicals and inflammatory responses; these indirectly result in increased numbers of alveolar macrophages that cause damage to lung tissues. This research was done by comparing a group of experimental animals (male Wistar rats) that were exposed to e-cigarette smoke to a control group.

Methods

In this study conducted between March and September 2019 in Airlangga University, male Wistar rats were used because there was't menstrual cycle in the subjects, which signifies no changes in hormone levels. During care and maintenance of experimental animals, the 3R principle of replacement, refinement and reduction was applied. The animals were kept in accordance with standard natural conditions of two cycles (12 hours of light and 12 hours of dark) and provided with healthy food and water.

E-cigarettes were used in this study, each containing 6 mg of nicotine. The liquid was heated using a coil made of nickel. The experimental method involved streaming smoke into a smoking chamber sized 70 × 50 × 30 cm. The rats were included in the chamber only when an appropriate smoke exposure had been given in accordance with the experimental duration. Malondialdehyde, IL-8, IL-10, metalloprotein-8 matrix and type-2 collagen require an immunohistochemistry (IHC) staining method for assessment. IHC assesses the expression of each sample quantitatively. The values are the average numbers of positive cells observed for each preparation. The data for each sample is the average value of the immunoreactive score observed in 10 different fields of view (LP) using a magnification of 400× on a light microscope.

The histological examination started with the removal of pulmonary organs of the rats to make preparations for the Hematoxylin-Eosin staining method. The assessment involves calculating the mean number of alveolar macrophages in 10 visual fields per preparation. Observations were carried out under objective magnifications of 40× using an immersion microscope.
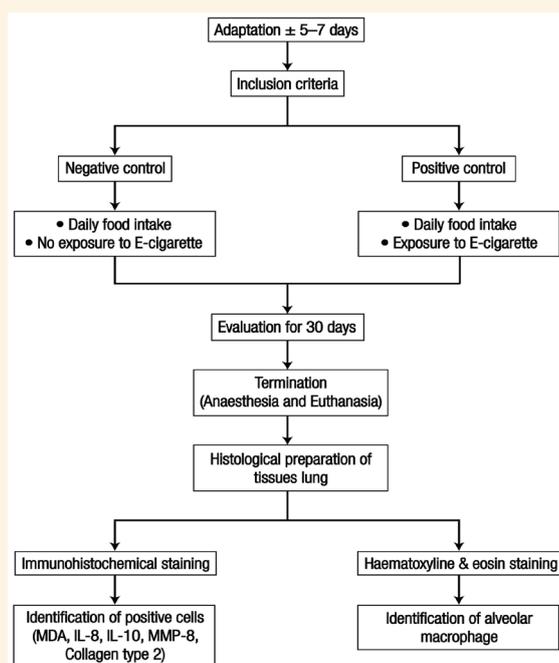

Figure 1: The methodology flowchart of an experimental study conducted on male Wistar rats assessing the levels of malondialdehyde (MDA), interleukin (IL)-8, IL-10, matrix metalloprotein-8 (MMP-8) and type-2 collagen following electronic-cigarette smoke exposure.
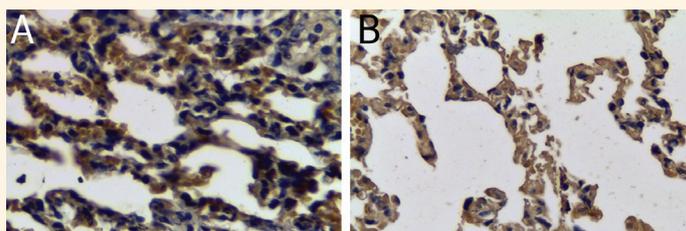
Figure 2: Immersion microscopic scans at 100x magnification showing a higher number of cells positive for malondialdehyde as seen in (**A**) the experiment group compared to (**B**) the control group of male Wistar rats based on positive antigen-antibody reactions expressed through the brownish-black cytoplasm.
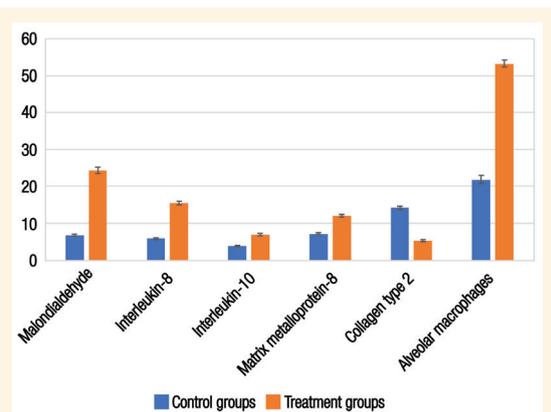

Figure 3: The mean value and standard deviation as seen in the experimental group compared to the control group of male Wistar rats in a study assessing the levels of malondialdehyde, interleukin (IL)-8, IL-10, matrix metalloprotein-8 and type-2 collagen following electronic-cigarette smoke exposure.

In this study, the experiment was divided into two groups. The first was the control group, which was given only food without any exposure to e-cigarette smoke. The second group was given food and exposed to e-cigarette smoke for two minutes daily during the 4-week period of the experiment [Figure 1].

The results of this study were obtained via a ratio data focused on the average number of positive cells from malondialdehyde, IL-8, IL-10, matrix metalloprotein (MMP)-8 and type-2 collagen in the lung tissue as well as the mean number of alveolar macrophages in each group. Data analysis was performed via t-test analysis using Statistical Package for the Social Sciences (SPSS), Version 22.0 (IBM Corp., Armonk, New York, USA). The next stage of the data analysis was performed using the partial least square (PLS) test to observe the effects between the variables.

This study received ethical approval from the Health Research Ethics Committee, Faculty of Public Health, Universitas Airlangga.

Results

The results of this study were obtained by comparing the average number of positive cells in 10 varying LPs between the control and experiment groups for malondialdehyde, IL-8, IL-10, MMP-8 and type-2 collagen. The mean value and standard deviation of each variable in both groups are definite. The t-test revealed a significant difference in the number of positive cells between the control and experiment groups [Figures 2a, 2b and 3].

In the experiment group, the number of positive cells from malondialdehyde (24.30 ± 0.82), IL-8 (15.49 ± 0.56), IL-10 (6.88 ± 0.35) and the MMP-8 matrix (12.01 ± 0.35) were significantly higher than those in the control group from malondialdehyde (6.80 ± 0.28), IL-8 (5.86 ± 0.27), IL-10 (3.91 ± 0.20) and the MMP-8 matrix (7.14 ± 0.23). Additionally, the number of positive cells of type-2 collagen in the control group (14.19 ± 0.47) was significantly higher than those in the experiment group (5.26 ± 0.27) [Figure 3].
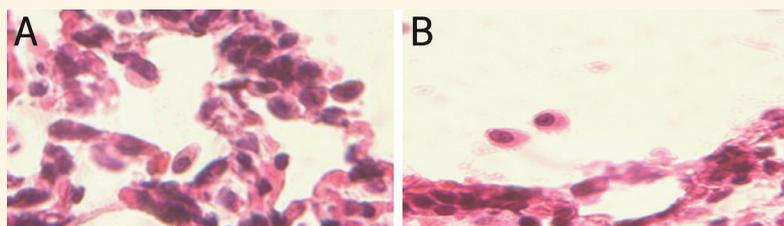

Figure 4: Immersion microscopic scans at 100x magnification showing a higher number of alveolar macrophage cells as seen in (**A**) the experimental group as compared to (**B**) the control group of male Wistar rats in a study assessing the levels of malondialdehyde, interleukin (IL)-8, IL-10, matrix metalloprotein-8 and type-2 collagen following electronic-cigarette smoke exposure.

Table 1: The results of the correlation-test for each group of male Wistar rats in a study assessing the levels of malondialdehyde, interleukin (IL)-8, IL-10, matrix metalloprotein-8 and type-2 collagen following electronic-cigarette smoke exposure

Protein	MDA*	IL-8[†]	IL-10[‡]	MMP-8[§]	Collagen type-2
IL-8[†]	0.995	-	-	-	-
IL-10[‡]	0.980	0.983	-	-	-
MMP-8[§]	0.989	0.991	0.977	-	-
Collagen type-2	−0.993	−0.992	−0.977	−0.991	-
Alveolar macrophage	0.995	0.995	0.994	0.994	−0.996

*MDA = malondialdehyde; [†]IL-8 = interleukin-8; [‡]IL-10 = interleukin-10; [§]MMP-8 = matrix metalloprotein-8.

The results of this study were obtained by comparing the mean number of alveolar macrophages per preparation in 10 LP between the control and experiment groups [Figures 4a and 4b]. The standard deviation and average value of alveolar macrophages were observed for each group. T-test results exhibited a difference in the number of alveolar macrophages between both groups (P <0.001), where the experiment group (53.26 ± 0.93) had a greater number of positive cells compared to the control group (21.86 ± 1.12) [Figure 3].

The results of the correlation test uncovered a strong association between malondialdehyde, IL-8, IL-10, matrix MMP-8, type-2 collagen and alveolar macrophages present in lung tissue (r >0.80) [Table 1].

Discussion

This research shows an increase in various parameters that can impact health negatively. It has been observed that the content found and inhaled from e-cigarettes will cause lung tissue damage. Free radicals passing through the airways trigger oxidative stress and set off lipid peroxidation, increasing the levels of malondialdehyde, as shown in the immunohistochemical micrographs [Figures 2 and 4]. E-cigarette smoke that enters and passes through the airway will increase the number of free radicals.[15] When these free radicals become excessive or abundant, oxidative stress also increases, which causes further lipid peroxidation and cell damage.[16] The product of a lipid peroxidation reaction is malondialdehyde, which is one of the final metabolic by-products of peroxidase and hence, a commonly used marker of increased free radicals in the body.[17] The increase in malondialdehyde levels in the experiment group in this study was due to free radicals from e-cigarettes entering the respiratory tract.

Cell damage causes cell necrosis, which in turn results in cellular debris, often called damage-associated molecular patterns (DAMPs) in the micro-environment.[18] Debris of cells stimulate the body's immune system by increasing the number of alveolar macrophages in the lung tissues. The first stage of immune defence in the body is carried out by M-1 alveolar macrophages through the process of phagocytosis.[19] The debris of cells that have been phagocytosed by these macrophages will stimulate the secretion of various cytokines that help initiate an inflammatory reaction, one of which is IL-8.[20] Whenever these reactions occur excessively, one of the M-2 macrophage cells will play a role in regulating the inflammatory process. Macrophage cells express IL-10, which acts as an anti-inflammatory agent.[21] The results of this study showed an increase in levels of interleukin-8 and a decrease in levels of interleukin-10 in the treatment group. This change in interleukin levels would cause an excessive inflammatory reaction in the lung tissue.

The increased inflammatory response in lung tissue in the experiment group stimulates alveolar macrophage and the secretion of metalloprotein-8 matrix, which is formed from the degranulation of polymorphonuclear neutrophils (PMNs) during the inflammatory reaction.[22] Non-PMN cells that experience these reactions, such as epithelial cells of the human bronchi, also produce a metalloprotein-8 matrix. This demonstrates the influence of the inflammatory process on the increase in metalloprotein-8 matrix in lung tissues.[22] An excessive increase stimulates the degradation of type-2 collagen tissue found in the cells. This type of collagen is the main active protein in cartilage tissues. In the airways, it is abundant in the trachea, until it reaches the small bronchial tubes. Increased synthesis and secretion of type-2 collagen causes structural and functional disorders of cartilage. Inflammation due to foreign particles damages the bronchial wall and is one of the risk factors of pulmonary fibrosis.[23] The increase in alveolar macrophage, metalloprotein-8 matrix and type-2 collagen that occurred in the experimental group of the current study can increase the risk of fibrosis in the lung tissue.

The body's ability to regulate inflammation is mainly through the secretion of anti-inflammatory cytokines, namely IL-10. These cytokines are produced by T helper 2 cells, which inhibit the activation of T helper 1 cells that play a role in increasing the number of macrophages in the lung tissue.[24] In addition, IL-10 is also needed to inhibit pathogen clearance and improve immunopathological conditions.[25,26] This was evidenced in the current study by the increase

of various parameters of the inflammatory response in the group exposed to e-cigarette smoke, which was contrasted with the control group results. Each parameter shows a relationship in the inflammatory response process caused by exposure to e-cigarette smoke, which forms a molecular pathway for lung tissue damage.

Conclusion

Exposure to e-cigarette smoke increases free radicals in the airways through increased malondialdehyde. This causes modifications in pro-inflammatory cytokines found in the lung tissue, such as IL-6 and IL-8, resulting in changes in the metalloprotein-8 matrix and commencing type-2 collagen degradation. Hence, this study showed a molecular pathway through its short-term negative impact caused by e-cigarette smoke that modifies the inflammatory process in the lung tissue and triggers the onset of lung tissue damage.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHORS' CONTRIBUTION

Both authors contributed equally to data collection, analysis and drafting the manuscript. Both authors reviewed the results and approved the final version of the manuscript.

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