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LUNG HISTO-PATHOLOGICAL CHANGES AND SODS GENE EXPRESSION IN WISTER RATS EXPOSED TO CONVENTIONAL AND ELECTRONIC CIGARETTES

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Background: *With the rapid increase in the use of electronic cigarettes as a prevalent form of nicotine, numerous studies have reported that their use has potentially negative health effects.* ***Objectives:*** *Evaluate and compare the effects of electronic cigarettes and traditional cigarettes on lung tissue.* ***Study Design and Methodology:*** *Twenty-five male Wistar rats were split into three groups: the EC Group was treated to e-cigarette liquid vapor, the CC Group to conventional smoke, and the control group without nicotine.* ***Result:*** *Significant alterations were observed in both experimental groups., including an increase in thickness of the alveolar wall, infiltration of inflammatory cells, collapsed vacuole in the wall of alveoli, and focal infiltration of lymphocytes in the wall of the bronchiole. Additionally, there was up-regulation of SODs gene expression in both groups.* ***Conclusion:*** *Histo-pathological analysis suggests that smoking and vaping are equally harmful to lung tissue. Real-time PCR analysis revealed significant up-regulation of SODs gene expression in conventional cigarettes and electronic cigarettes compared to the control group*

Keywords: *E-cigarette, C-cigarette, lung, Histopathology, SODs genes*

INTRODUCTION

Smoking is a well-known and established risk factor for several health conditions, including lung cancer, heart disease, stroke, respiratory illnesses, and other chronic illnesses. The negative impact of smoking on health has been documented through numerous studies and research over the past decades. The tobacco industry has also been subject to increased regulation and taxation in many countries to reduce the prevalence of smoking and its associated health risks. Additionally, public health campaigns have been launched to educate people about the dangers of smoking and encourage them to quit. Despite continuous efforts, smoking remains a significant public health concern globally, and efforts to reduce its prevalence and negative impact on health continue to be a priority. For decades, the adverse consequences of smoking have been recognized. Chronic active and passive smoking raises the risk of pulmonary diseases, fibrosis of the lungs, asthma, malignancies, cardiovascular diseases, and metabolic disorders[1&2]. Nicotine, the most biologically

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active component of cigarette smoke, is primarily responsible for cigarette smoking's adverse effects[1]. Because nicotine receptors are distributed throughout the body, the effects on human health are complex[1,3]. The lungs are responsible for absorbing most of the inhaled nicotine into the bloodstream, resulting in a significant impact of nicotine on lung cells[4&5].

Cigarette smoking is considered the leading cause of oxidative species' high concentration; smoking increases the production of reactive oxygen species (ROS) which is a major source of oxidative stress[6]. ROS interacts with molecules, including carbohydrates, proteins, lipids, and nucleic acids. These interactions cause damage or change the function of the target molecule. Additionally smoking disrupts the natural balance between oxidation and antioxidation reactions, increases superoxide anion generation and induces oxidative stress. Studies indicate a rise in mRNA expression of superoxide dismutase (SOD) enzymes. The antioxidant enzyme SOD neutralized the effect of ROS by catalyzing the conversion of superoxide anions to hydrogen peroxide[7]. Three SODs exist: copper zinc SOD (CuZnSOD), manganese SOD (MnSOD), and extracellular SOD (ECSOD). It is believed that cytosolic CuZnSOD (SOD1) is the primary superoxide radical scavenger in the cytosol and nucleus of the cell. CuZnSOD levels are high in many cells, and CuZnSOD transgenic animal studies have concluded that the protection provided by CuZnSOD is not significantly improved by CuZnSOD overexpression[8], and the natural intracellular levels of CuZnSOD are sufficient under the majority of pathophysiological conditions. MnSOD (SOD2) is a mitochondrial enzyme known to play an important role in the oxidant resistance of several vital organs[8&9]. MnSOD overexpression protects alveolar epithelium from oxidative stress, whereas MnSOD deficiency is fatal; MnSOD knockout mice die within 1–2 weeks of birth[9]**.** ECSOD (SOD3) is a tetrameric protein containing Cu and Zn that is composed of two dimers. Other antioxidants can adequately compensate for the absence of ECSOD in mice, and ECSOD-deficient mice appear to develop normally, unless they are subjected to extreme stress. In these deficient animals, ECSOD has been demonstrated to play a significant role in lung protection during inflammatory states[10].

E-cigarettes are commonly introduced as a safe alternative to tobacco smoking. The vapor generated by electronic cigarettes (e-cigs) are non-combustible nicotine along with propylene glycol, vegetable glycerin and a broad range of flavors. This is why it is believed to be a safer form of 'smoking,' particularly in the treatment of cigarette addiction[11]. E-cigarette liquids contain various substances that, when heated, produce aerosol (vapor) that mimics smoke[12&13].

Inconsistent data exist regarding the safety of e-cigarette use, although initially it was believed that the use of e-cigarettes was completely safe, further observations revealed that vaporizers, like smokers, suffer from a variety of disorders; consequently, additional research was conducted[14-16]. People who use e-cigarettes have experienced pain, dizziness, fever, vomiting, gingivitis, cough, sore throat, and severe organizing pneumonia with hypoxemic respiratory failure[17&18].

In addition to formaldehyde, carbonyls, and nitrosamines found in conventional cigarettes, Additional toxic substances may be contained in vapor[6.] These detrimental effects may also be caused by trace metals such as nickel, chromium, tin, and aluminum, which are all leached from the e-cigarette's core assembly[19&20].

The current study hypothesized that Users of e-cigarettes may experience clinical signs of lung function issues due to histological changes in lung tissue. Using an animal model, this study aimed to compare the safety of electronic cigarettes to that of conventional cigarettes.

Study design

From July 10 to September 10 of 2021, this study was carried out in the School of Medicine at Wasit University. Animals were weighed weekly throughout the experiment, starting off at a weight of about 170 grams. They were then sorted into three groups: EC Group (10 rats) received 1cc of liquid vapor of E-cigarette (daily dose 3 mg nicotine), CC Group (10 rats) received 10 C-cigarette (daily dose 3 mg of nicotine), and Control Group (5 rats) without receiving any nicotine exposure.

The Biomedical ethics committee at the college of medicine /Wasit University

approved the protocol for this study with ethical approval number **(UW. Med. 2023.074)**.

Rats in the experimental groups were kept in a smoking room for one hour each day for 45 days.

The CC group was given cigarettes (KENT 0.3 mg Nicotine; International). Electronic cigarettes (Voopoo Drag Max 177W Pod Mod KIT) were chosen for the experiment because they are the most popular brand and employ fluid with nicotine concentrations of 3 mg/ml.

Apparatus

The smoking room in the current study was converted from an anaerobic box chamber. The box utilized in this project was created for the local market using the specifications for the chamber that Montanari and Christian had devised in 2020, which were as follows: sealed exposure chambers were modified standard rat cages (259mmX234mmX209mm) to deliver vaporized nicotine and conventional cigarette smoke into the chambers. To supply nicotine vapor puffs from both electronic and traditional cigarettes to each chamber, MedPC IV software activated three e-Vape generator controllers set at 4.5 watts **(Fig. 1)**. To maintain an air flow rate of around 3.5 L/min, the air chamber was vacuum-controlled. Nicotine was blended with the surrounding air stream and fed to the chambers through polyethene tubing attached to the cartridges after the generator controls were activated. Vacuum air pumps attached to the chambers sucked smoke and nicotine vapor out of the chamber and into the exhaust[21].

Tissue collection and staining

Animals were anaesthetized using Inhaler Anesthetic Liquid "Inhale Solution" and then sacrificed by decapitation according to the Ministerial procedures approved for the species. Then lung tissue was collected and kept in a container that had 10% formalin. After that histological preparation of lung tissue and staining by Hematoxylin and eosin stain was done and a light microscope was used to examine the slides of lung tissue. Additionally, lung samples were also taken and preserved in -80^{o}c refrigerator (deep freeze) for Real - time PCR analysis.

Real- time PCR analysis

Total RNA was isolated from rat lung using total RNA Purification Kit following the (Thermo Scientific, Fermentas, #K0731) according to the manufacturer's instructions. Using a spectrophotometer (ND-1000 spectrophotometer) the ratio of absorbance values at 260/280 nm was calculated to determine the RNA's purity. cDNA was then synthesized using (Thermo Scientific, Fermentas, #EP0451) according to the instructions provided by the manufacturer. The cDNA products (1µL) were used as a template for each polymerase chain reaction (PCR) amplification. Real-time PCR was carried out as described in[22]. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as the internal reference[23], and order-specific primer designs for SOD1, SOD2, SOD3, and GAPDH were designed by[24]. As shown in **Table (1)**, these primers were supplied by (Bioneer company, Korea).

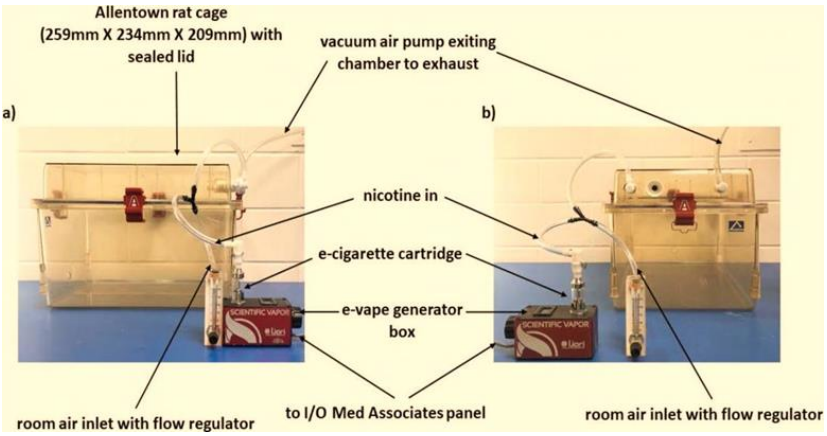

Fig. 1: Front (panel a) and side (panel b) views of the model of the apparatus were modified in the present study (Montanari, Christian, et al 2020).

Table 1: The sequence of primer pairs used in this study.

Gene name	Orders	Annealing temperature
SOD1	Forward: 5'AATGTGTCCATTGAAGATCGTGTGA3'	60C
	Reverse: 5'GCTTCCAGCATTTCCAGTCTTTGTA3'	
SOD2	Forward: 5'AGGGCCTGTCCCATGATGTC3'	63C
	Reverse: 5'AGAAACCCGTTTGCCTCTACTGAA3'	
SOD3	Forward: 5'GGGTCTGTCCTGTACTTCACCAGAG3'	60C
	Reverse: 5'CTGACATGGTCCAGGTGACAGAG3'	
GAPDH	Forward: 5'GCACCGTCAAGGCTGAGAAC3'	60C
	Reverse: 5'ATGGTGGTGAAGACGCCAGT3'	

A SYBR Green Real-time PCR kit was used (AccuPower ® Greenstar TM qPCR PreMix 96 plate, Bioneer, Korea). The volume of the reaction system was 20 ml and it contained 0.2 mM of upstream primer, 0.2 mM of downstream primer, 10 ml of SYBR Premix Taq, 7.2 ml of purified water, and 2 ml of cDNA. The two-step PCR reaction procedure consisted of pre-denaturation at 95uC for 30 seconds, a PCR reaction at 95uC for five seconds, and 45 cycles at 60 or 63uC for twenty seconds. This reaction was carried out with the aid of an Exi-cycler Real-Time PCR Amplifier. (Bio-rad China).

The relative quantification gene expression levels (fold change) Livak method outlined by Livak and Schmittgen, was used to analyze the q RT-PCR data for the target gene and the housekeeping gene[25].

Table 1. The sequence of primer pairs used in this study.

Statistical analysis

The picture from all three groups (control, conventional cigarette, and electronic cigarette) was analyzed by counting lymphocyte numbers per section (H&E, x40). Couniting of lymphocytes was done by using ImageJ 1.05b, Wayne Rasband, National Institute of Health, USA. Then the significance was done by applying students' t-test to the data. The p-value was then calculated, and significance was considered if $p \leq 0.05$.

RESULTS AND DISCUSSION

Results

Histo-pathological study

The present study shows a normal wall of alveoli and bronchiole representing the normal tissue obtained from the control group **(Fig. 2A)**, while the tissue of rats which were exposed to conventional cigarettes, shows an increase in thickness of the alveolar wall, infiltration of inflammatory cell, collapsed and vacuole in the wall of alveoli **(Fig. 2B)**, and the same signs almost has been noticed in lung tissue of rats exposed to E. cigarette. Moreover, there was hyperplasia in the wall of the alveoli, vacuole and collapsed alveoli wall **(Fig. 2C)**. While in conventional cigarettes significant signs have been reported in the wall of alveoli, the vacuole which was fully by blood (Fig. 2D), where it has not been observed in the vaping group **(Fig. 2E)**. The present study also noted that, in conventional cigarettes there is an increased thickness of the bronchiole wall and focal infiltration of lymphocytes in the wall of the bronchiole **(Fig. 2F)**.

Investigation of inflammatory cell infiltration (lymphocyte) in three groups of the present study; control, conventional cigarette, and the electronic cigarette revealing a significant change in cell number per section stained with H&E and investigated in light microscope on x40 **(Fig. 3)**. The mean number of lymphocytes in the control group was 340 cells/section, and it was elevated significantly in EC ($p \leq 0.001$) where it reaches 569

cells/section. The number of lymphocytes in the CC group was also higher (737 cells/section) compared to the control group with a significant difference ($p \leq 0.001$). There was also a significant difference ($p = 0.0025$) between CC and EC in the number of lymphocytes where the CC group has a higher number.

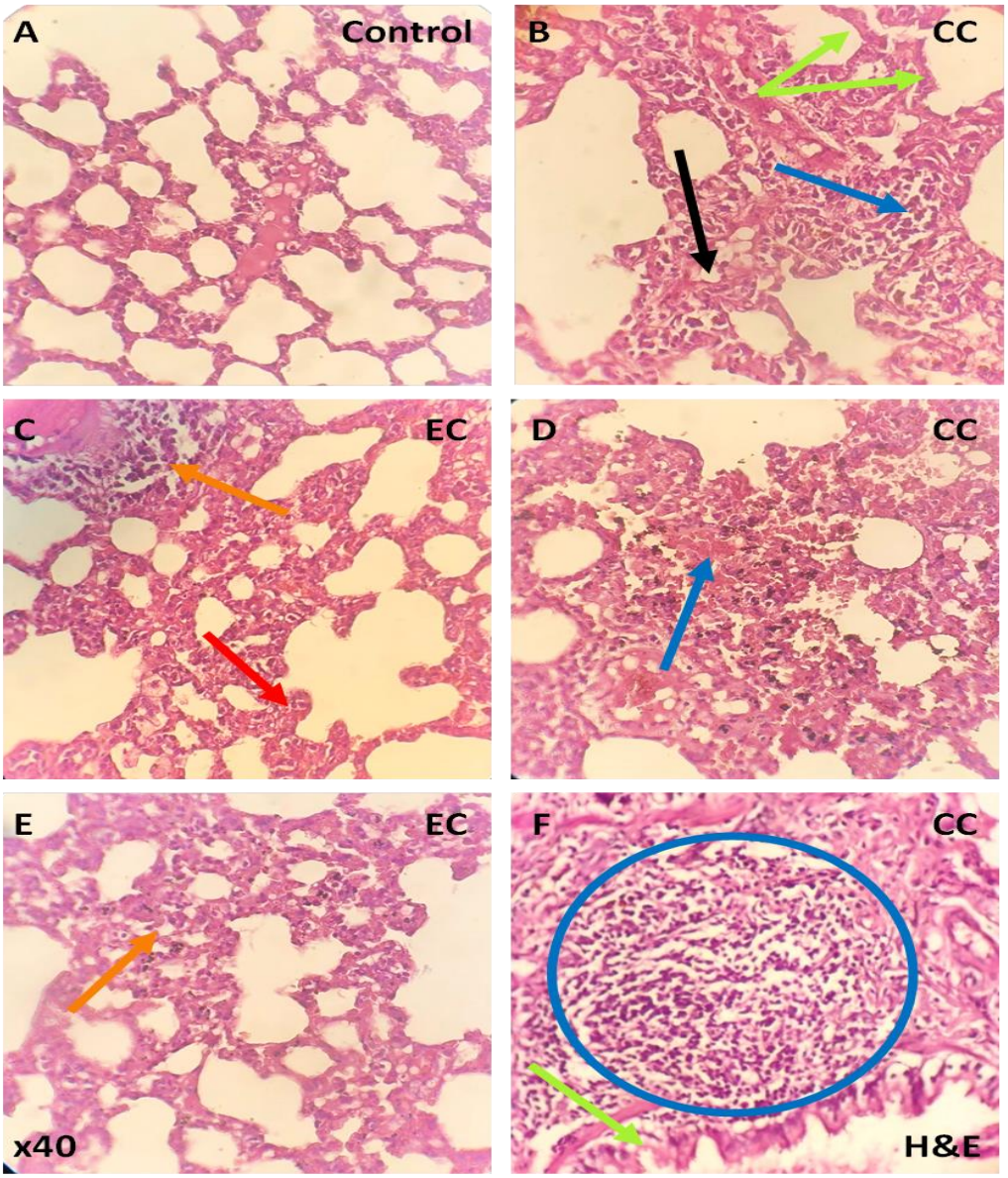

Fig. 2: Lung tissue is obtained from the rat of three groups, control, conventional cigarette (CC), and electronic cigarette (EC). All tissue was stained with H&E and investigated on x40. A) Normal histological lung of control rats. B) Lung tissue of CC, there was an increase in thickness of the alveolar wall (green arrow), inflammatory cell infiltration (blue arrow) and vacuole (black arrow). C) Lung tissue of EC, show hyperplasia of the alveolar wall (red arrow) and inflammatory cell infiltration (orange arrow). D) Lung tissue of CC, there is a vacuole, and some are filled with blood (blue arrow). E) Lung tissue of EC, there is a vacuole in the wall of alveoli without blood (orange arrow). F) Green arrow represents the increase in thickness of the bronchiole wall for lung tissue CC and focal infiltration of lymphocytes in the wall of l bronchiole (blue circle).

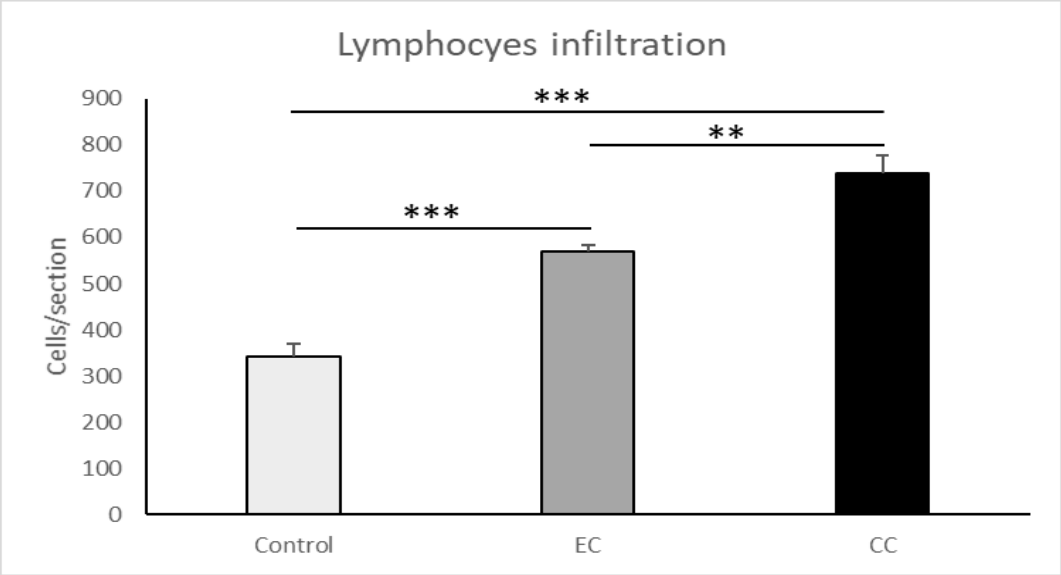

Fig. 3: The number of lymphocytes infiltrating lung tissue, cells per section, in the control, EC, and CC groups. EC: electronic cigarette, CC: conventional cigarette. *** $p \leq 0.001$, ** $p \leq 0.01$. Analyzed sections were on 40x.

Real- time PCR analysis

In the present study, Quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed to determine the changes in SODs gene expression. We found that the expression of SODs mRNA was significantly higher in the E-cigarette and C-cigarette groups compared to the control group. **(Fig. 4)**.

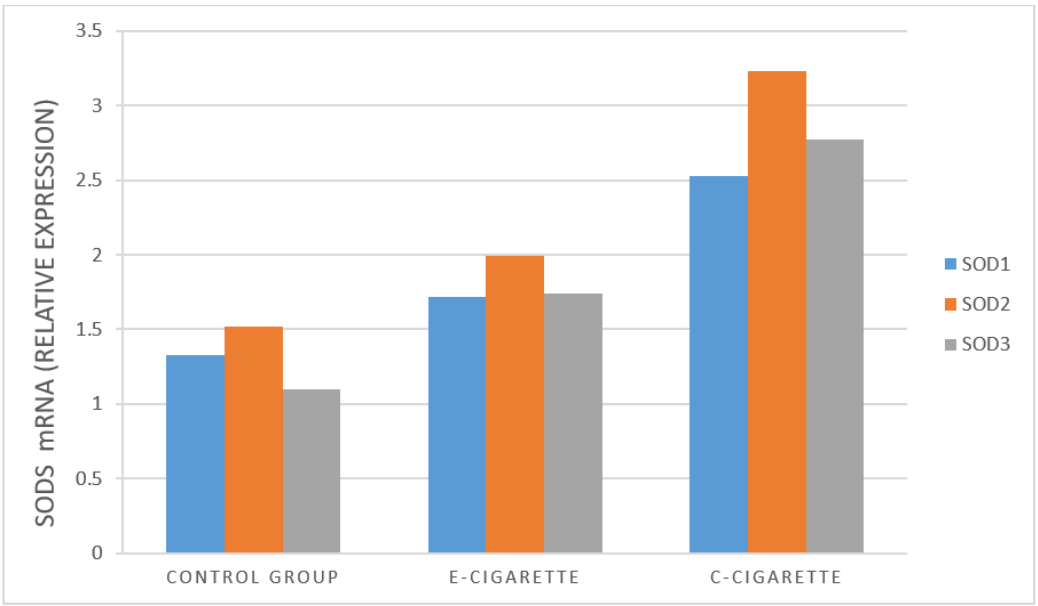

Fig. 4: E-cigarette groups and C-cigarette groups increased SOD1, SOD2 and SOD3 gene expression. Total RNA was extracted from the lungs of each animals group and qRT-PCR was performed using specific for SOD1, SOD2 and SOD3 gene. GAPDH was used as an internal control gene. The changes in mRNA levels are displayed as the gene's fold expression in the test sample relative to its expression in the control group .Results are expressed as the means ± SD, n = 3. * $p < 0.01$.

The mRNA expression of SOD1, SOD2, and SOD3 was up regulated in the E-cigarette group by 0.29, 0.31, and 0.58-fold, respectively, compared to the control group ($p < 0.05$). In contrast, the mRNA expression of SOD1, SOD2, and SOD3 increased significantly 0.91, 1.125, and 2.51-fold, respectively, in the C-cigarette group ($p < 0.01$).

Moreover, the upregulation of SODs gene expression was significantly higher in C-cigarette groups than E-cigarette groups ($p < 0.01$).

GAPDH was used as an internal control gene. The changes in mRNA levels are displayed as the gene's fold expression in the test sample relative to its expression in the control group .Results are expressed as the means ± SD, n = 3. * $p < 0.01$.

Discussion

Although the assumption is that vaping e-cigarettes is safe and promoted as a healthy alternative to cigarette smoking, public health specialists have offered differing opinions also there are conflicting data regarding their safety and usefulness as smoking cessation tools[26].

In this study, the alveolar wall of rats shows that there is an increase in the thickness, and infiltration of lymphocyte cells in the wall of alveoli and collapsed due to the toxic effect of nicotine and other chemicals, in both conventional and E. cigarette and these lead to the irritation of alveolar wall and increase the thickness also affects the function of the lung during gas exchange. These results were consistent with those of another study carried out by Ziad S. et al. in 2013[27] in which it was shown that rats exposed to conventional cigarettes showed a distinct thickening of the alveolar wall tissue. The study discovered that the collapse of alveoli, which caused a reduction in the efficiency of gaseous exchange in alveoli and identified inflammatory cell infiltration with blood extravasations, was a characteristic in the lung tissue impacted by a conventional cigarette.

Ewelina Wawryk Gawda et al. (2020)[28] discovered parenchymal collapse, hyperphagia, and type II pneumocyte hyperplasia. Both smoking and vaping cigarettes resulted in an increase in macrophages, however the current study reveals that the traditional cigarette group exhibits considerable blood extravasation and blood-filled vacuoles in the alveolar wall. This result may be due to the toxic effect of free radicals in traditional cig. which causes damage to the wall of a blood vessel, and this leads to the exudation of blood from the blood vessel. Other researchers agreed with this assessment (Rivan V. Suryadinata and Bambang Wirjatmadi. 2021)[29] and asserted that e-cigarette use causes a considerable rise in free radical concentrations of 1016 molecules per puff or more, which contributes to cellular damage and rupture. Church and Daniel F,1985 also confirm these findings and state that cigarette smoke contains two different populations of free radicals in both tar and gas-phase smoke and has toxic effects on lung tissue[30].

In the current study, conventional cig. show focal infiltration of lymphocytes in the wall of the bronchiole and increase the thickness of the wall, while these signs do not appear in the bronchiole of rats exposed to E. cigarettes.

The result of this study real –time PCR revealed that, the mRNA expression of SOD1, SOD2, and SOD3 was up-regulated in the E-cigarette and C- cigarette groups compared to the control group. Similar results were obtained by Russo et al.[31] who observed that chronic continuous exposure of free radicals increase antioxidant enzyme gene expression, Stringer et al.[32] similarly reported an increase in SODs in rats chronically exposed to cigarette smoke.

Chronic exposure to high levels of free radicals may result in up-regulation of antioxidant enzyme gene expression. This hypothesis appears to be supported by studies of Harju *et al* and Jenifer *et al* , that demonstrate an increase in superoxide dismutase enzyme levels in the airways of smokers[33&34]. Moreover, the up-regulation of SODs gene expression were significantly higher in C-cigarette groups compared to E-cigarette groups ($p < 0.01$) this significant up-regulation in SODs maybe due to its higher ROS contents and this finding could explain the severe changes in lung histopathological structure of c-cigarette group than e-cigarette groups .

Conclusion

Our findings indicate that the harmful effect of electronic cigarettes on lung samples was almost like that of tobacco cigarettes compared to healthy animals, furthermore, with conventional cigarette exceeds its effect to reach the bronchiole, there is focal infiltration of lymphocyte and increase thickness of the wall and signs in the wall of alveoli. There is extravasation of blood and filling some vacuoles by blood but still has not been proven to us through this study that the use of electronic cigarettes is safer. However, our previous studies that relied on the questionnaire for electronic cigarette smokers often indicated that it is a safer way to quit smoking permanently in a short time. There is still a need to introduce more long-term studies and detect heavy metals formed because of using electronic cigarettes and their toxic effect on the lungs.

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التغيرات في الأنسجة الرئوية والتعبير الجيني SOD في فئران الوستر المعرضة للسجائر العادية والإلكترونية

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لعقود من الزمان ، تم التعرف على الآثار السلبية للتدخين. يزيد التدخين المزمن من مخاطر الإصابة بأمراض الرئة وتليف الرئتين والربو والأورام الخبيثة وأمراض القلب والأوعية الدموية واضطرابات التمثيل الغذائي.

السجائر الإلكترونية ، المعروفة باسم vaping ، منتشرة بشكل متزايد كشكل من أشكال استخدام النيكوتين. أفادت العديد من الدراسات أن استخدام السجائر الإلكترونية يمكن أن يساهم في إدمان النيكوتين وله آثار صحية سلبية محتملة بينما اكدت دراسات اخرى ان التدخين الالكتروني يمتلك ضررا اقل من التدخين التقليدين واعتبر البعض ان الاخير يمكن ان يعد طريقة للإقلاع عن التدخين بكل أنواعه.

يحتوي دخان السجائر على تركيز عالٍ من الأنواع المؤكسدة ؛ يزيد التدخين من إنتاج أنواع الجذور الحرة (ROS) والتي تعد مصدرًا رئيسيًا للإجهاد التأكسدي. من المعروف أن أنواع الجذور الحرة (ROS) لها تأثيرات ضارة يتم تحييدها بواسطة مضادات الأكسدة. ومن مضادات الاكسدة هي انزيمات (SODs)superoxide dismutase والتي بدورها تقلل الجذور الحرة (ROS) عن طريق تحفيز تحويل الأنيونات الفائقة(superoxide anions) إلى بيروكسيد الهيدروجين.

افترضت الدراسة الحالية أن مستخدمي السجائر الإلكترونية قد يعانون من علامات سريرية لمشاكل وظائف الرئة بسبب التغيرات النسيجية في أنسجة الرئة. لذا تم تقييم الضرر الحاصل على انسجة الرئة باستخدام نموذج حيواني ، هدفت هذه الدراسة إلى مقارنة سلامة السجائر الإلكترونية بسلامة السجائر التقليدية ثم تم تأكيد ما وجد عن طريق التعبير الجيني لجين superoxide dismutase والذي يعتبر المسول المباشر عن معاملة الجذور الحرة والتخلص منها.

تصميم التجربة وطريقة العمل

تم تقسيم خمسة وعشرين من ذكور جرذان Wistar إلى ثلاث مجموعات لمدة ٤٥ يوم: تم معالجة مجموعة EC على بخار سائل السجائر الإلكترونية ، ومجموعة CC للدخان التقليدي ، ومجموعة السيطرة بدون التعرض لأي مما سبق.

بعد ذلك تم التحضير النسيجي لأنسجة الرئة والتلطيخ بواسطة صبغة الهيماتوكسيلين والأيوزين وتم استخدام مجهر ضوئي لفحص شرائح أنسجة الرئة. بالإضافة إلى ذلك ، تم أخذ عينات من الرئة وحفظها في ثلاجة −٨٠ من اجل الحصول على RNA.

الجهاز

تم تصميم صندوق التدخين المستخدم في الدراسة الحالية من السوق المحلية بطريقة مشابهه لما مستخدم في تجربة مونتاناري وكريستيان في عام ٢٠٢٠ ، والتي كانت على النحو التالي: تم تعديل صناديق الفئران القياسية (٢٥٩ ملم × ٢٣٤ ملم × ٢٠٩ ملم) لتوصيل النيكوتين المبخر ودخان السجائر التقليدية. لتزويد كل غرفة ببخار النيكوتين من السجائر الإلكترونية بالضافة الى السكائر التقليدية.

النتائج

تظهر أنسجة الفئران التي تعرضت للسجائر التقليدية زيادة في سمك الجدار السنخي ، وتسلل الخلايا الالتهابية ، وانهيارها وفجوة في جدار الحويصلات الهوائية ، وقد لوحظت نفس العلامات تقريبًا في أنسجة الرئة في الفئران التي تعرضت للسجائر الالكترونية. لاحظت الدراسة الحالية أيضًا أنه في السجائر التقليدية هناك زيادة في سمك جدار القصبات وتسلل بؤري للخلايا الليمفاوية في جدار القصبات. تظهر السيجارة التقليدية ارتشاحاً بؤرياً للخلايا الليمفاوية في جدار القصبات وتزيد من سمك الجدار ، بينما لا تظهر هذه العلامات في القصبات الهوائية للفئران المعرضة للسجائر الإلكترونية.

تم إجراء تحليل (RT-PCR) لتحديد التغيرات في التعبير الجيني لـ SODs. وجدنا أن التعبير عن SODs mRNA كان أعلى بشكل ملحوظ في مجموعات السجائر الإلكترونية والسجائر التقليدية مقارنة بمجموعة السيطرة للأنواع الثلاثة من SODعلى حد سواء. كذلك وجد هنالك زيادة واضحة في مجموعة التدخين التقليدي بالمقارنة بالتدخين الالكتروني.

قد تكون هذة الزيادة في المستوى الجيني لل SODs بسبب ارتفاع محتويات الجذور الحرة(ROS) وقد تفسر هذه النتيجة التغيرات النسيجية المرضية لمجموعة السجائر التقليدية أكثر شدة من مجموعات السجائر الإلكترونية

الخلاصة

تشير النتائج التي توصلنا إليها إلى أن التأثير الضار للسجائر الإلكترونية على عينات الرئة كان تقريبًا مشابه لتأثير سجائر التبغ مقارنة بالحيوانات السليمة ، مع ذلك قد تجاوز تأثير السجائر التقليدية في اغلب الحيوانات للوصول إلى القصبات الهوائية، كما ان هناك تسلل بؤري للخلايا اللمفاوية وزيادة سمك الجدار، وعلامات في جدار الحويصلات الهوائية وهناك تسرب للدم وملء بعض الفجوات بالدم ومع ذلك لم يثبت لنا من خلال هذه الدراسة أن استخدام السجائر الإلكترونية أكثر أمانًا. بالإضافة الى ما سبق ، فإن دراساتنا السابقة التي اعتمدت على الاستبيان الخاص بمدخني السجائر الإلكترونية أشارت غالبًا إلى أنها طريقة أكثر أمانًا للإقلاع عن التدخين بشكل دائم في وقت قصير من خلال عدد الذين اقلعوا عن التدخين.