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Nicotine in Vapor Exposure Decreases Hippocampal Neurons in Rats

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Abstract

Background: Recently, many people have considered electronic cigarettes to be a safer alternative to conventional tobacco cigarettes, even though they still contain harmful substances such as nicotine, which is associated with a decrease in hippocampal neurons. **Objective:** This study aimed to examine the effect of exposure to e-cigarettes on hippocampal neurons in rats. **Methods:** An experimental study was performed using 26 tissue samples obtained from Wistar rats. The rats were randomly assigned to four groups and treated over an eight-week period as follows: the control group (K) (n=7) had no vapor exposure; the V0 group (n=6) received vapor without nicotine (0 mg/ml); the V6 group (n=7) was exposed to vapor and 6 mg/ml nicotine; and the V12 group (n=6) was exposed to vapor and 12 mg/ml nicotine. Hippocampal neurons were examined using hematoxylin-eosin staining at 400x magnification across 7 fields of view. Data were analyzed using the Kruskal-Wallis test with the Mann-Whitney post-hoc. **Results:** The number of hippocampal neurons was significantly reduced in both the V12 and V0 groups compared to that in the control group ($p<0.05$). The lowest number of hippocampal neurons was observed in the V0 group (0 mg/ml nicotine), followed by the V12 group (12 mg/ml), and then the V6 group (6 mg/ml).
Conclusion: The present study showed that the variation of nicotine in vapor exposure decreases hippocampal neurons in rats.

Keywords: Electronic cigarettes, nicotine, hippocampal neurons.

Original Research Article

INTRODUCTION

Over the past decade, the use of electronic cigarettes (ECs) and vaporizers has surged, particularly among teenagers and adults (Pesko & Robarts, 2017). This increase is often due to the belief that ECs are "cool," safer, and healthier alternatives to traditional cigarettes (CCs) (Brikmanis et al., 2017; Fauzi & Areesantichai, 2022). According to the 2021 Global Adult Tobacco Survey (GATS), e-cigarette usage increased from 0.3% in 2011 to 3% in 2021, with approximately 68 million users globally (Jerzyński et al., 2021).

However, vaping can be addictive and harmful Because of its liquid components, including propylene glycol (PG), vegetable glycerin (VG), carbonate compounds, metallic residues, nicotine, and flavoring agents. These substances can cause neurotoxicity, stimulate dopamine release, and activate the reward system through aerosolizations (Yuan et al., 2015; Walley et al., 2019).

In vivo studies have shown that nicotine exposure at specific doses can cause inflammation and neuronal death in various brain regions, such as the prefrontal cortex and parts of the hippocampus, including the dentate gyrus and cornu ammonis 1 and 3 (Chen et al., 2021; Wijaya et al., 2022; Kuncorowati et al., 2020). Neuronal cell death, or necrosis, is marked by the loss of membrane integrity, leakage of intracellular substances, and activation of inflammatory cytokines, which can disrupt cognitive, motor, and short-term memory functions (Prasedya et al., 2020). Nicotine exposure from electronic cigarettes has been found to increase the expression of α4-6β2-containing neuronal nicotinic acetylcholine receptors (nAChRs) in various brain regions, including the central nervous system, ventral tegmental area (VTA), nucleus accumbens (NAC), prefrontal cortex (PFC), and hippocampus. This upregulation affects the reward system, leading to addiction and neurotoxicity due to cholinergic over-stimulation (Bird, 2015; Ruszkiewicz et al., 2020).

Research on the effects of nicotine on neurons, particularly in the hippocampus, a region vital for cognitive functions and memory, is limited (Kaisar et al., 2016). The hippocampus is essential for creating and updating relational memory, which is crucial for adaptive thinking and social interactions. Research shows that damage to this brain region can lead to rigid and inappropriate behaviors, especially when tasks require generating, recombining, and flexibly applying information. This impact is evident across various functions, including memory, navigation, exploration, imagination, creativity, decision-making, evaluating character, forming and sustaining social connections, empathy, communication, and language. Therefore, the hippocampus, along with its broad network of connections with other brain systems, supports the flexible application of information (Rubin et al., 2014). A previous study indicated a reduction in the neuron count after nicotine exposure at doses of 0 and 18 mg/ml (Wu et al., 2020; Chen et al., 2021). Another study examined how exposure to electronic cigarettes (ECs) affects hippocampal neuron morphology compared to conventional cigarette (CC) exposure, finding signs of atrophy and increased levels of inflammatory cytokines (TNF-α and IL-6) through immunohistochemical staining (Prasedya et al., 2020). However, this study did not quantify neuronal morphology or explore its correlation with nicotine concentration and was limited to a single dose. Consequently, further research is urgently needed to explore how different nicotine concentrations affect the number of hippocampal neurons, which are crucial for cognitive, memory, and motor function.

MATERIALS AND METHODS

Study Design

This research has received ethical approval from the ethics committee of the Faculty of Medicine, Universitas Islam Indonesia (certificate number: 16/Ka.Kom.Et/70/KE/XII/2023). The sample gained from the previous study had a true experimental design with a randomized control group and a post-test-only design. The number of samples was determined using the resource equation formula (Arifin & Zahiruddin, 2017), n = DF/k + 1. Based on the acceptable range of the DF, the DF in the formulas was replaced with the minimum (10) and maximum (20). The minimum number of animals per group: n = 10/k + 1. The Maximum number of animals per group was = 20/k + 1. where N = total number of subjects, k = number of groups, and n = number of subjects per group.

Twenty-eight male Wistar rats (Rattus norvegicus) aged 4-5 weeks old weighing 100 ± 20 g were obtained from Laboratorium Penelitian dan Pengujian Terpadu, Universitas Gadjah Mada, Yogyakarta, Indonesia. The rats were acclimatized for seven days under a light/dark cycle of 12 h at 22 ± 2 °C and 50 ± 10% humidity, and placed in a cage with two rats each. The rats were then randomly divided into four groups: Control group (K) that did not receive vapor and nicotine (n=7), V0: received vapor and 0 mg/mL nicotine concentration in EC liquid (n=6), V6: received vapor and 6 mg/mL nicotine

concentration in EC liquid (n=7), and V12: receive vapor and 12 mg/mL nicotine concentration in EC liquid (n=6). The rats were fasted for 12 h after eight weeks of exposure and continued to be terminated by injected with 50-75 mg/kg of Zoletil 50 (25 mg/mL tiletamine hydrochloride and 25 mg/mL zolazepam hydrochloride) intramuscularly for anesthesia. The brain was collected for Hematoxilin-Eosin staining in the Laboratorium Penelitian dan Pengujian Terpadu, Universitas Gadjah Mada, Yogyakarta, Indonesia, for a month and continued for a histological study by 2 observers in the Histology and Pathology Anatomy Laboratory, Faculty of Medicine, Universitas Islam Indonesia for two weeks. We included intact brain samples that could be read clearly with a microscope at various magnifications and excluded damaged samples with incomplete coloring.

Measurement of Neuron

Observations of the preparations were carried out at the Histology Laboratory of the Faculty of Medicine, Islamic University of Indonesia, with two observers. The reading began with initial observations to determine the eligibility of the observed slides. If the slides met the inclusion criteria, the preparations was further observed. The numbers of CA1, CA2, and CA3 hippocampal pyramidal cells were observed using a light microscope connected to an analysis application on a computer (Oyem & Odokuma, 2018). Observations of the preparations used a magnification of 400x in seven fields of view per preparation with details of the CA1 region taken 2-3 fields of view, the CA2 region taken in two fields of view, and the CA3 region taken 2-3 fields of view (Arjadi et al., 2014). The criteria for observing normal neurons included neurons in the pyramidal cell layer with a round central vesicular nucleus with prominent nucleoli. The cytoplasm contained prominent basophilic Nissl cytoplasmic granules surrounded by a thin neuropil.

Data Analysis

Data analysis began by measuring the level of agreement (>0,50) between assessors using the Interclass Correlation Coefficient (ICC). The number of CA1, CA2, and CA3 hippocampal pyramidal cells was analyzed using the Kruskal-Wallis statistical test and Mann-Whitney post-hoc for pairwise comparison with a 95% confidence level (α=0.05). The level of significance was determined at $p < 0.05$.

RESULTS

Interclass Correlation Value

This study used biological materials stored in rat hippocampal tissue from previous studies totaling 26 pieces. The data were validated by an anatomical pathologist expert from the Department of Histology and Anatomical Pathology, Faculty of Medicine, Universitas Islam Indonesia calculating reliability values using interclass correlation (ICC) in Table 1. The ICC value was obtained from the comparison (equation variance) / (total variance) = (equation variance)/ (equation variance + undesirable variance) (Liljequist et al., 2019). The mean agreement score was 0.918 (see Table 1), which signified the researchers' inter-rater agreement and excellent consistency. Based on the validity tests, the data on hippocampal neurons in male Wistar rats exposed to vapor demonstrated high validity and reliability

Table 1. The reliability value using interclass correlation (ICC)

		95% Confidence Interval		*F Test with True Value 0*			
	Intraclass Correlation	***Lower Bound***	***Upper Bound***	**Value**	**f1**	**f2**	**Sig**
Single Measures	.918	.825	.962	23.299	25	25	.000
Average Measures	.957	.904	.981	23.299	25	25	.000

Hippocampus Neuron Measurement Result

The average number of neurons shown in Figure 1 indicated that EC exposure in K group is 89.14±52.638; in V0 group is 8.14±7.403; in V6 group was 39.67±31.538; and in V12 group was 13.5±4.970. A subsequent normality test indicated a non-normal distribution ($p<0.05$) in the 6 mg vape group (V6) (see Figure 1).

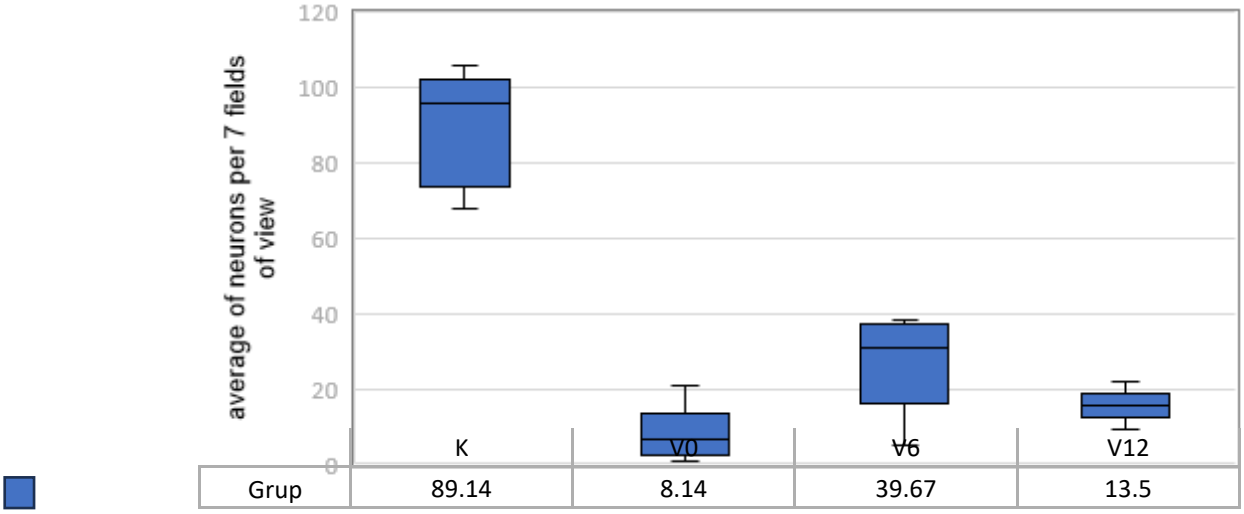

Figure 1. The average number of hippocampus neuron

The data were analyzed using the Kruskal-Wallis test as the One-Way ANOVA assumptions were not eligible. The Kruskal-Wallis test showed a significant p-value ($p<0.05$), specifically $p=0.014$, indicating a statistically significant reduction in neuron count with varying nicotine concentrations from vapor exposure. The Mann-Whitney U test further calculated the differences between groups, finding significant differences in neuron counts between groups K vs. V0, K vs. V12, and V0 vs. V6 (refer to Table 2). No significant differences were detected between groups K vs. V6, V0 vs. V12, and V6 vs. V12. The group with the lowest average hippocampal neuron count was V0 (8.14), whereas group V6 (39.67) had the highest average count.

Table 2. Mann-Whitney U test results

Kelompok	K	V0	V6	V12
K	-	-	-	-
V0	0.032*	-	-	-
V6	0.084	0.022*	-	-
V12	0.031*	0.294	0.061	-

Hippocampal Histology Analysis

The analysis of tissue samples showed a decrease in the number of neurons in the hippocampal CA1, CA2, and CA3 regions in the V0, V6, and V12 groups compared to the control group. Hematoxylin and eosin (H&E) staining of the hippocampus in the control group displayed typical neuronal features, including uniformly sized layers of pyramidal cells arranged in a regular pattern. Each neuron had a centrally located vesicular nucleus with a distinct nucleolus, and its cytoplasm contained basophilic Nissl granules, all surrounded by a thin neuropil. In contrast, the V0, V6, and V12 groups exhibited pyramidal neuronal necrosis, including pyknotic nuclei (shrunken, dense, and irregular) and eosinophilic cytoplasm (see Figure 2).

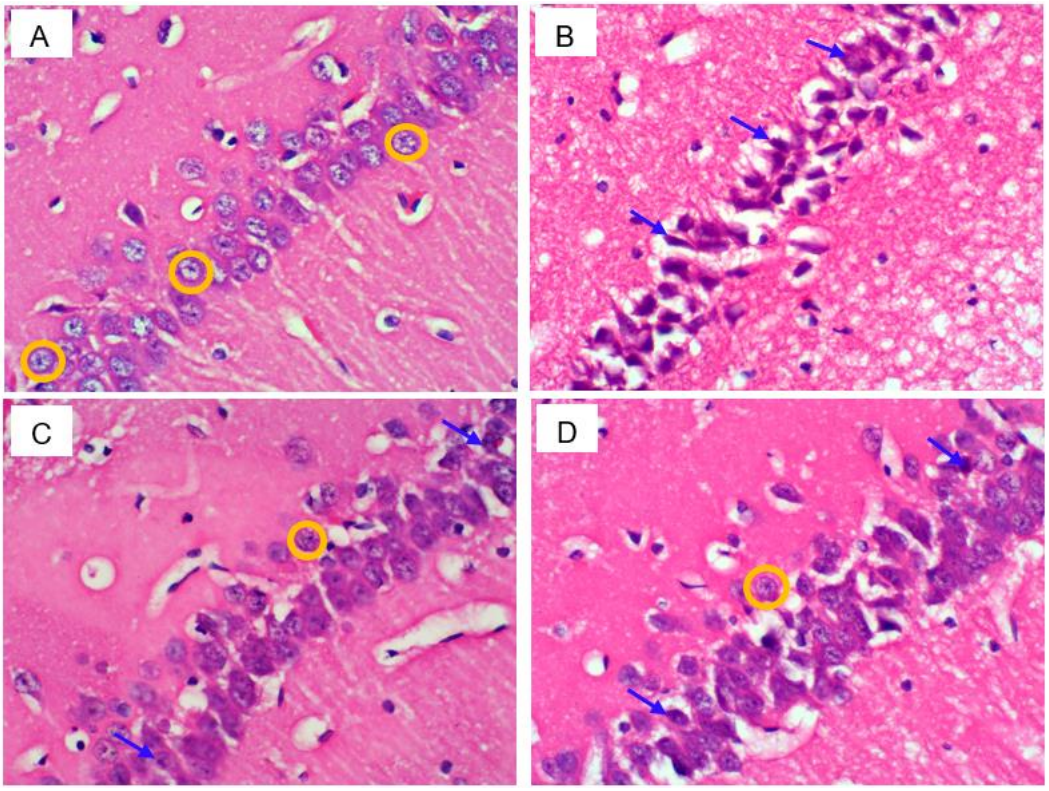

Figure 2. Histology of the hippocampus with H&E staining at 400x magnification. The hippocampus of the control group (A) showed normal pyramidal cells, as indicated by yellow circles. In the hippocampus of groups, V0 (B), V6 (C), and V12 (D), blue arrows indicate pyramidal cells with pyknotic nuclei (condensed and angular) accompanied by eosinophilic cytoplasm, indicating necrosis.

DISCUSSION

This study found that vapor exposure significantly reduced the number of hippocampal neurons at nicotine concentrations of 12 and 0 mg/ml. However, at a concentration of 6 mg/ml, the decrease in hippocampal neurons did not achieve statistical significance compared to the control group. These findings align with those of a previous study that observed even nicotine vapor at 0 mg/ml (PG/VG 50%/50%) significantly reduced hippocampal neuron counts ($p<0.05$). Higher nicotine concentrations (18 mg/ml) further diminish hippocampal neuron numbers (Chen et al., 2021). Low-dose nicotine 6.3 mg/kg/day had no effect on cognitive function and did not decrease bdnf expression in the striatum. High-dose nicotine 18 mg/kg/day decreased cognitive function and inhibited bdnf expression in the striatum. Bdnf is a protein that functions in neuroplasticity and memory function (Ortega et al., 2013). Similarly, the present study reported hippocampal neuron damage from nicotine exposure, though with different doses and methods, showing increased damage at nicotine doses of 2 mg/kg and 4 mg/kg administered via subcutaneous injection compared to control and 0.25 mg/ml groups (Ijomone et al., 2015). Hippocampal neuronal damage from e-cigarette exposure, though specific nicotine doses have not been studied in detail (Wijaya et al., 2022). Low-dose nicotine vapor exposure (0 mg/ml and 3 mg/ml) over 30 days led to increased necrotic pyramidal neurons in the prefrontal cortex compared to the control group, suggesting that each gram of nicotine could increase pyramidal neuron necrosis by 1.6 times (Kuncorowati et al., 2020).

Toxic effects arise from the combustion of liquids that contain not only nicotine but also several harmful substances, producing free radicals, including aldehydes, formaldehyde, acetaldehyde, and acrolein (Traboulsi et al., 2020). Among these, acrolein notably increases NADPH oxidase 2 (Nox-2) levels and stimulates the production of reactive oxygen species (ROS) in endothelial cells, leading to a decrease in neuronal counts (Kuntic et al., 2020; Qiu et al., 2016). ROS levels from vapor-liquid combustion are positively related to vegetable glycerin (VG) concentration and battery power (Haddad

et al., 2019). ROS activates microglia, reduces glucose transporter 1 (Glut1) levels, and disrupts tight junction proteins (Occludin), causing neuronal damage and a decline in neuron numbers (Heldt et al., 2020). Additionally, metal compounds found in vapor, such as arsenic (As), cadmium (Cd), lead (Pb), chromium (Cr), copper (Cu), and nickel (Ni), have neurotoxic effects that impair neurotransmitter release and cause mitochondrial damage (Gaur & Agnihotri, 2019). Consequently, even vapor with a nicotine concentration of 0 mg/ml can lead to neurotoxic effects, particularly affecting the hippocampus and cognitive functions, memory, and emotional regulation (Alzoubi et al., 2021).

The toxic effects of nicotine are mediated through the activation of nicotine adenine dinucleotide phosphate (NADPH) oxidase (NOX), which leads to metabolic imbalances and cell death (Noda & Kobayashi, 2017). Activation of NOX-2 and NOX-4 in the hippocampus reduces the production of superoxide (O2-), affecting the levels of nitric oxide (NO) and peroxynitrite (ONOO). This superoxide reduction increases the oxidation of tetrahydrobiopterin (BH4), leading to a deficiency in BH4, a co-factor for nitric oxide synthase (NOS). This disruption in endothelial nitric oxide synthase (eNOS) binding results in decreased NO production by the vascular endothelium, causing vasoconstriction and subsequent hypoperfusion, which reduces the supply of oxygen and glucose. The diminished availability of glucose and oxygen, which are crucial for brain energy metabolism, leads to metabolic dysfunction and triggers neuronal death (Han et al., 2021). At higher doses, such as 18 mg/ml in vapor, nicotine can induce neuronal necrosis as part of an inflammatory response (Prasedya et al., 2020).

Shrunken nuclei, eosinophilic cytoplasm, and vacuolization characterized neuronal damage in groups V6 and V12. However, the reduction in neuron count in the V6 group was not significantly different from that in the control and V12 groups. Nicotine can exert neuroprotective effects, although this is not the case at high doses (Dong et al., 2017; Tizabi et al., 2021). Oral nicotine at a dose of 6 mg/kg/ml for 42 days significantly increased hippocampal neuron count (Oyem & Odokuma, 2018). Other studies have also indicate that nicotine may have neuroprotective benefits, such as increasing dopamine levels in Parkinson's disease when administered in diluted forms (Carvajal-Oliveros et al., 2021; Nicholatos et al., 2018; Tizabi et al., 2021). These varying outcomes could be due to differences in nicotine dosage, administration methods, and exposure durations, suggesting that while 6 mg/ml nicotine still led to decreased hippocampal neurons in this study, the effect was not significantly different from the control group.

CONCLUSION

The present study shows that variation of nicotine in vapor exposure decreases hippocampal neurons in rats. Exposure to nicotine vapor at doses of 0 and 12 mg/ml reduces the count of normal neurons in the hippocampus of male rats. Based on the study's limitations, comparing different hippocampal regions due to their distinct functions is recommended. Similar studies should investigate the effects of varying nicotine levels and the classification of hippocampal structural damage in each region, as well as ensure consistency between the right and left sides.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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