

# DECREASED PURKINJE

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**Decreased density of pyramidal cells in the cerebral cortex, and Purkinje cells in the cerebellar cortex of Sprague-Dawley rats after being exposed to filtered kretek cigarette smoke**

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**Contributions:** conceptualization: DT, EP, and HA. Data acquisition: DT, and EP, Data analysis or interpretation: EP, HA, HJE. Drafting of the manuscript: DT, EP and HA. Critical revision of the manuscript: EP, HA, HJE, RAD, and AVO. Approval of the final version of the manuscript: all authors.

**Ethics approval:** This research has received a certificate of passing the ethical review from the Research Ethics Commission of the Faculty of Medicine, Universitas Trisakti, with No: 184/KER/FK/VIII/2018.

**Introduction**

Smoking globally causes considerable health problems. Around 2.5 billion people in the world are smokers and that number even greater is in third-world countries. Indonesia's

population is included in the group of third-world countries, meaning that a consistent portion of the population consumes cigarettes.<sup>1</sup>

Cigarette smoke is a gas that contains solid materials (particulates), including carcinogenic ingredients. Moreover, it is stated that tobacco has a significant negative impact on the development of nervous structures<sup>2</sup> and causes addiction.<sup>3</sup> Gases and particulates of cigarettes are channeled into the alveoli.<sup>4</sup> It is also stated that smoking affects the activity and function of the brain and interferes with the psychology of cigarette addicts.<sup>5</sup>

The cerebral cortex is the brain's largest and most visible component; it is separated into right and left hemispheres by a deep groove or fissure known as the major longitudinal fissure.<sup>6</sup> The cerebral cortex's surface is extended by multiple gyri, which are elevations separated by grooves called sulci.<sup>7</sup> Neurons in the cerebral cortex have pyramid-shaped cell bodies. The cerebral cortex is grey matter on the outside of the cerebral hemispheres, and the centre of the medulla is white matter on the inside of the cerebral hemispheres.<sup>6</sup> Myelinated and unmyelinated fibres, oligodendrocytes, fibrous astrocytes, and microglial cells are all found in white matter. Perikaryon or nerve cell bodies, unmyelinated fibres, myelinated fibres, astrocytes, oligodendrocytes, and microglial cells are all found in grey matter. The quantity of myelinated nerve fibres is indicated by the unique colour of the white matter.<sup>7</sup>

The cerebral cortex serves as a hub for learning, memory, sensory integration, information processing, and motor response initiation. This structure is made of grey matter and contains an estimated 10 billion nerve cells.<sup>9</sup> Efferent pyramidal cells are the most common nerve cells and can be seen under a microscope.<sup>10</sup>

The results of previous studies showed that cigarette smoke is a risk factor for the occurrence of aneurysms in the brain (cerebral aneurysms). It has also been reported that cigarette smoke increases the risk of rupture.<sup>11,12</sup> It was further stated that more than 80% of patients with Aneurysmal Subarachnoid Hemorrhage (ASH) had a history of smoking, and 50–60% were smokers.<sup>11</sup> So far, it is not known how filtered kretek cigarette smoke affects the biometrics of brain cells.

Previous research has shown the importance of cell biometrics as a characteristic that needs to be developed to assist in early diagnosis at the cellular and tissue levels.<sup>13,14</sup> This needs to be done so that the diagnosis related to cell abnormalities is determined more objectively based on the quantitative data obtained and not only qualitative data. The brain

is the focus of research because of the importance of this organ in the regulation of body activities. In addition, parts of the brain, namely the cerebral cortex and cerebellum, are vulnerable to exposure to foreign substances.<sup>15,16</sup> Cigarette smoke mostly contains alkaloids. The results of previous studies have shown that cigarette smoke is associated with decreased cognitive function and causes dementia. More precisely, cigarette smoke causes thickening of the cortex in the brain.<sup>17</sup> Another study has demonstrated that cigarette smoke inhibits mitochondrial respiration in the rat brain.<sup>18</sup> To explore more deeply the effect of cigarette smoke on the density of pyramidal cells in the cerebral cortex, and Purkinje cells in the cerebellar cortex, we used Sprague-Dawley rats as animal models. Our recent research demonstrates the importance of cell biometrics as a characteristic that needs to be developed to aid early diagnosis at both cellular and tissue levels.<sup>19,20</sup>

## **Materials and Methods**

### ***Research design***

This study used *Rattus norvegicus*, Sprague-Dawley strain, to test the effect of filtered kretek cigarette smoke on pyramidal cells in the cerebral cortex and Purkinje cell density in the cerebellar cortex. This study has two groups, the treatment group and the control group, thus adopting an experimental and control group design.

### ***Location and time of research***

Experimental animal treatment was carried out at the RSHP Laboratory of the Faculty of Veterinary Medicine, IPB-University, Bogor, Indonesia. Histological observations of the preserved preparations of rat brains (cerebral and cerebellar cortex) were carried out at the Biology Laboratory, Faculty of Medicine, Universitas Trisakti, Jakarta, Indonesia. This research was conducted between September 2021 to June 2022. This research has received a certificate of passing the ethical review from the Research Ethics Commission of the Faculty of Medicine, Universitas Trisakti, with No: 184/KER/FK/VIII/2018.

### ***Selecting and grouping of experimental animals***

*Rattus norvegicus* Sprague-Dawley strain rats, aged 2-3 months with a body weight of 200-300 g, were included in the study; the exclusion criteria were if the animals died during

treatment. The number of samples is estimated in accordance with World Health Organization (WHO) guidelines, which indicate that at least 5 animals must be utilized in experimental research. Each set of six rats was employed in this study. The control group is group 1, while the treatment group is group 2.

#### ***Treatment of experimental animals***

Rats were acclimatized for one week before the trial began. During acclimation, rats were provided food and water. Rats were not treated during acclimation. After acclimation, the groups were randomised at random. Group 1 (6 rats) is the control group, and the rats breathe normally. Group 2 (6 rats) was exposed to filtered kretek cigarette smoke 1 stick every day for 3 months (90 days).

Filtered kretek cigarette smoke exposure was carried out in a smoking chamber measuring 45 x 35 x 20 cm (31,500 cm<sup>3</sup>). The oxygen valve was opened, then the cigarette was attached to the pipe connected to the pump, then the cigarette was burned and the pump was turned on so that the smoke will enter the smoking chamber and be inhaled by the rats. Filtered kretek cigarette smoke exposure was carried out on group 2 for 10 minutes every day in the morning.

The rearing cage measures 482 x 267 x 210 mm (27025740 mm<sup>3</sup>=27025.740 cm<sup>3</sup>). The treatment cage room was equipped with air conditioning with a temperature of 22 ± 3°C, humidity 55 ± 5%, and LED lights (12:12 hours, light and dark cycle), feed and drink in normal amounts (reasonable). The standard feed used for rats was brailler-II pellet (BR-II) containing corn, soybean meal, wheat pollard, coconut meal, fish meal, meat meal, rice flour, tapioca, coconut oil, and fish oil premix. Feed and water were provided *ad libitum*. Feed was given as much as 10% body weight (± 10-15 grams day), every day in the morning and evening. Drinking water for rats was always changed every day.

After treatment, the rats were anesthetized until they died. Anesthesia was administered using ketamine 100 mg/kg body weight and xylazine 10 mg/kg body weight intraperitoneally. After the treatment, the experimental animals were sacrificed by euthanasia, then their organs, namely the brain, were taken. To take the rat brain, decapitation was carried out, then the skull and brain were separated. The brain that had been separated from the skull, was then put into a small pot containing 10% neutral buffered formalin (NBF).

### ***Observation of rat cerebral and cerebellar tissues***

The thickness of the cerebral tissue, as well as the cerebellar prepared for making slides, was 3 mm. Rat cerebral tissue, as well as rat cerebellar tissue in paraffin blocks were cut with a thickness of 5  $\mu$ m. Next, the tissue sections were stained with hematoxylin and eosin (HE). Observations were made on pyramidal cells in the cerebral cortex, in the hippocampus area. In addition, Purkinje cells were also observed in the gray matter of cerebellar cortex. Observation of pyramidal cells and Purkinje cells was carried out by 3 observers according to predetermined guidelines.

### ***Data analysis***

The statistical test chosen in this study was an independent statistical test T-test to compare the histometric brain cells of rats between the group 1 and the group 2. Differences between groups were significant if the results of the analysis showed a p value <0.05.

### ***Results***

Brain morphology of Sprague-Dawley rats and sites of tissue sectioning for preparation of slides from the cerebral and cerebellar tissues are presented in Figure 1.

Photomicrograph of rat cerebral cortex in group 1 and group 2 are presented in Figure 2, while a comparison of the density of pyramidal cells in the cerebral cortex of rats in the hippocampal area between groups 1 and group 2 is presented in Figure 3.

Based on Figure 3 above, the distribution of pyramidal cells in the rats cerebral cortex at the hippocampus area of group 1 was normal, while group 2 showed a decrease in pyramidal cell density. Comparison of pyramidal cell indices in the rats cerebral cortex in the hippocampus area between group 1 and group 2 is presented in Figure 4.

The density of pyramidal cells in the rats cerebral cortex at the hippocampus area between the group 1 was compared to group 2 ( $p < 0.001$ ). The result of statistical analysis showed that t value=22.262; df=118; mean difference=7.60983; SE diff.=0.34182; 95% CI=6.93293-8.28674.

Photomicrograph of the rats cerebellar cortex is presented in Figure 5 and Figure 6.

1 Purkinje cells in the rats cerebellar cortex in the group 1 seemed normal, while in the group 2 they appeared degenerated. Comparison of Purkinje cell distances in the rat cerebellar cortex between the group 1 compared to the group 2 is presented in Figure 7. Based on Figure 7, the distances of Purkinje cells in the rats cerebellar cortex of the group 1 were more dense than those in the group 2. There were differences in the distance of Purkinje cells in the rats cerebellar cortex between group 1 compared to the group 2 ( $p < 0.001$ ). The result of statistical analysis showed that  $t$  value = 29.263;  $df = 118$ ; mean diff. = 1.38517; SE diff. = 0.04733; 95% CI = 1.47890 to 1.29143.

### Discussion

Alkaloids are found in tobacco. Cigarettes on the market contain approximately 1.5% alkaloids by weight of tobacco. Tobacco, the primary component of cigarettes, accounts approximately 95% of the overall alkaloid content.<sup>21</sup> Nicotine ingested when smoking travels quickly to the brain. A prior study found that 2 mg of nicotine was absorbed systemically after 20 minutes of smoking and 80 puffs using a nicotine inhaler device.<sup>22</sup> In this study, filtered kretek cigarette smoke exposure can impact brain structure and function. Our study's approach of cigarette smoke exposure is similar to other studies.<sup>23</sup> It is indeed difficult to arrange for each rat to be exposed to the same amount of cigarette smoke. This can be remedied by repairing the smoking box.

Photomicroscopy of the cerebral cortex from the frontal lobes of rats revealed the pia mater, which encloses the molecular layer, followed by external granular, external pyramidal, internal granular, internal pyramidal, and a polymorphic innermost layer. Group 1 had a higher density of pyramidal cells in the exterior granular layer of the hippocampus than Group 2. We calculated the pyramidal cell density, often known as the pyramidal cell density index. This is accomplished by dividing the number of pyramidal cells by the length of a line passing through the exterior granular layer. The same study found that the control group had normal histology in the cerebral cortex layer, whereas tramadol administration caused pyramidal cell degeneration. Furthermore, granular cells with pyknotic nuclei and the formation of haloes.<sup>24,25</sup> Previous research found that tramadol administration caused histological abnormalities and apoptosis in the cerebral cortex,

which was linked to oxidative stress.<sup>26</sup> More specifically, the onset of apoptosis is preceded by damage to the DNA structure.<sup>27</sup>

Pyramidal cells in the rat cerebral cortex are easily recognized as the cells are quite large and in large numbers. In group 1, pyramidal cells in the rats' cerebral cortex appeared normal with high density. The density of pyramidal cells in the rats' cerebral cortex was lower in group 2 compared than the group 1. The results of previous studies demonstrated that pyramidal cell death can be caused by hypoxia,<sup>28,29</sup> so that the cells experience cell hemostatic disorders.<sup>30</sup>

Purkinje cells are found in the ganglion layer of the cerebellar cortex. Purkinje cells in the ganglion layer of the rats' cerebellar cortex seemed to be conical in form in group 1. Purkinje cells in the ganglion layer of the rat cerebellar cortex in group 2 were deteriorated and necrotic, according to our findings. Also, the vascularization is becoming increasingly thick. We believe this is owing to compensatory degeneration and necrosis, which is accompanied by enhanced anastomotic perfusion. According to the findings of this study, the position of Purkinje cells in the ganglion layer of the rats' cerebellar cortex in groups 1 and 2 did not change. This means that the Purkinje cells are still in the correct location in the ganglion layer. Purkinje cells in the ganglion layer of the rats' cerebellar cortex in group 2 were obviously pyramidal in shape, indicating degeneration and necrosis. It is widely assumed that cerebellar cognitive function involves interactions between the cerebellar cortex and cerebral cortex association areas.<sup>31</sup>

The cerebellar cortex is susceptible to toxic chemicals such as nicotine. Nicotine can cause Purkinje cells to experience mild, moderate and severe hyperplasia depending on the dose, time and method of administration.<sup>32</sup> Our findings contradict prior research that found nicotine to be protective in the cerebellar granule cells.<sup>33</sup> When rats were given nicotine for seven days, they developed modest hyperplasia compared to rats given nicotine for 21 and 42 days. Furthermore, it was indicated that exposure to modest dosages of nicotine for a brief length of time promoted neuronal cell growth.<sup>34</sup>

Our results are in line with previous studies were reported that nicotine causes neurodegeneration in the cerebellar cortex. This is due to the administration of higher doses of nicotine and a longer administration time.<sup>35</sup> Our results are also in agreement with previous studies which demonstrated that Purkinje cells undergo neurodegeneration due to



exposure to cigarette smoke in rats.<sup>36</sup> It should be noted from the results of previous studies that low doses of nicotine have a stimulatory effect on the central nervous system.<sup>37</sup> The findings of our study add to the evidence that low-dose filtered kretek cigarette smoke is harmful to the brains of Sprague-Dawley rats.

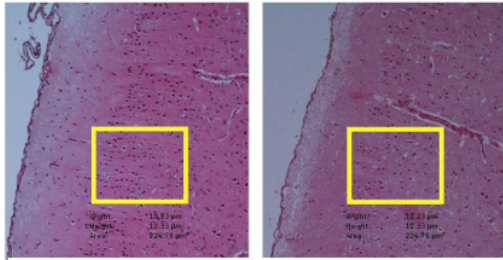
Despite the fact that we did not measure the levels of chemical compounds in filtered kretek cigarette smoke, which is a limitation of this study, the facts show that pyramidal cells in rats' cerebral cortex appear abnormal and have decreased in density. Furthermore, it was shown that Purkinje cells degenerate and their cell density decreases in the rat cerebellar cortex. Furthermore, we did not perform immunohistochemical analysis as a specific marker of various cytotypes in the cerebral and cerebellar cortex of rats.

### **Conclusions**

According to the study's findings, rats exposed to filtered kretek cigarette smoke 1 stick/day for 3 months reduced the number of pyramidal cells in the cerebral cortex around the hippocampus. Exposure to filtered kretek cigarette smoke at a rate of 1 stick/day for 3 months reduced Purkinje cell density in the cerebellar cortex.



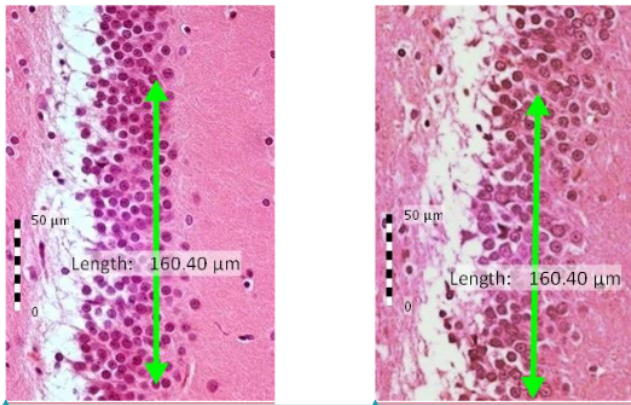
**Figure 1.** Brain morphology of Sprague-Dawley rats and sites of tissue sectioning for preparation of slides from the cerebral and cerebellar cortex. Red circle=bregma; white circle=lambda; black line=tissue cutting site for making cerebral slides; red line=tissue cutting site for making cerebellar slides.



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B.

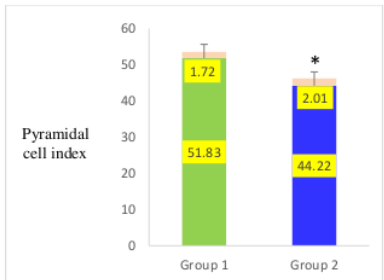
**Figure 2.** Photomicrograph of rat cerebral cortex, stained with hematoxylin and eosin (objective 10 X). Yellow rectangles are used to show the density of pyramidal cells. A. Cerebral cortex of rat in group 1. B. Cerebral cortex of rat in group 2.



Group 1

Group 2

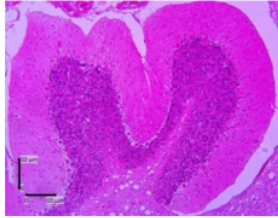
**Figure 3.** Hippocampus area of cerebral cortex in rat. A. Hippocampus area of cerebral cortex in group 1, stained with hematoxylin and eosin (objective 40 X). B. Hippocampus area of cerebral cortex in group 2, stained with hematoxylin and eosin (objective 40 X).



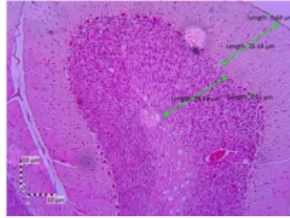
**Figure 4.** Comparison of pyramidal cell index in the hippocampus area of the rat cerebral cortex. \*=significant difference ( $p < 0.001$ ).

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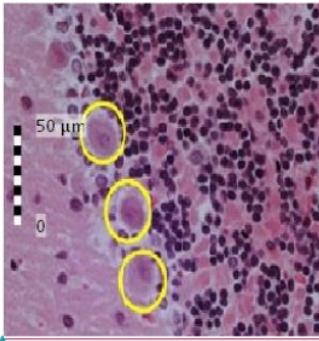


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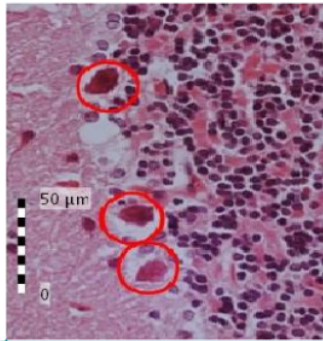


B.

**Figure 5.** Photomicrograph of rats cerebellar cortex. A. Photomicrograph of rat cerebellar cortex, stained with hematoxylin and eosin, objective 4 X. B. Photomicrograph of rat cerebellar cortex, stained with hematoxylin and eosin, objective 10 X.



A.

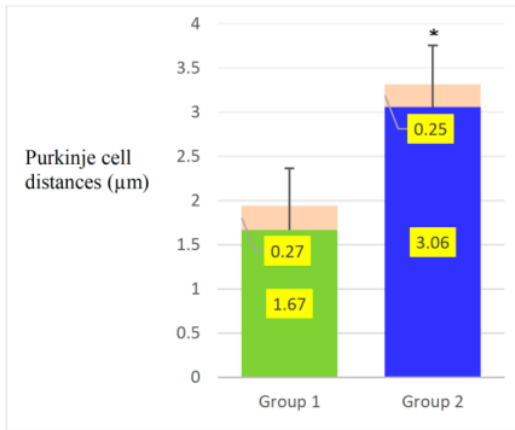


B.

**Figure 6.** Histological appearance of the rat cerebellar cortex. A. Rats cerebellar cortex in the group 1, stained with hematoxylin and eosin, objective 40 X. B. Rats cerebellar cortex in the group 2, stained with hematoxylin and eosin, objective 40 X. Purkinje cells in the rat cerebellar cortex in the group 1 looks normal (yellow circle), while in the group 2 there is a picture of degenerated Purkinje cells (red circle).

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**Figure 7.** Comparison of Purkinje cell distances in rat cerebellar cortex. \*=significant difference ( $p < 0.001$ ).

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