ORIGINAL ARTICLE

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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Abstract

Background: Filtered kretek cigarette smoke is in the form of gas containing solid materials (particulates). Such cigarette smoke contains carcinogenic substances. In addition, it is stated that tobacco has a significant negative impact on the development of neural structures, causes addiction, and affects brain activity and function.

Aim :of this paper was to determine the effect of filtered kretek cigarette smoke on the density of pyramidal cells in the cerebral cortex, and Purkinje cells in the rat cerebellar cortex.

Methods: The study was conducted experimental and control group design. Group 1 (6 rats) is the control group, the rats breathe using ordinary air. Group 2 (6 rats) is a group of rats exposed to filtered kretek cigarette smoke 1 stick/day for 3 months of treatment. Observations were made on pyramidal cells in the rat cerebral cortex, and Purkinje cells in the cerebellar cortexrat cerebellar.

Results: The distribution of pyramidal cells in the cerebral cortex atin the hippocampus area of rats in the group 1 was expected. In contrast, the group 2 showed a decrease in the density of pyramidal cells. There was a difference in the density of pyramidal cells in the rat cerebral cortex between the group 1 compared to the group 2 ($p<0.001$). Purkinje cells in the cerebellar cortexrat cerebellar in the group 1 were normal, while in the group 2 there was a picture of degenerated Purkinje cells. The distance between Purkinje cells in the cerebellar cortexcerebellar of the group 1 was denser compared to the group 2 ($p<0.001$).

Conclusion: Rats exposed to kretek cigarette smoke 1 stick/day for 3 months decreased pyramidal cell density in the hippocampus area of the cerebral cortex at the hippocampus area. The same thing happened: exposure to kretek cigarette smoke 1 stick/day for 3 months decreased Purkinje cell density in the cerebellar cortexrat cerebellar.

Keywords: cerebralcerebral cortex, and cerebellcerebellar cortexar, filtered kretek cigarette smoke, pyramidal cell, cerebellar Purkinje cell.

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Introduction

Smoking is a global problem that causes health problems. Around 2.5 billion people in the world are smokers and that number is in third-world countries. Indonesia's population is included in the group of third-world countries, meaning that many of the population consume cigarettes.[1]

Cigarette smoke is a gas that contains solid materials (particulates). In addition, cigarette smoke is also known to contain carcinogenic ingredients. Moreover, it is stated that tobacco has a significant negative impact on the development of nervous structures,[2] and cause addiction.[3] Gases and particulates of cigarettes are channeled into the alveoli.[4] In addition, it is also stated that smoking affects the activity and function of the brain and interferes with the psychology of cigarette addicts.[5]

The cerebral is the largest and most prominent part of the brain. The cerebral is divided into the right and left hemispheres by a deep groove or fissure called the major longitudinal fissure.[6] The surface of the cerebral is extended by many gyri, which are elevations and are separated by grooves called sulci.[7] The cerebral cortex has neurons with pyramid-shaped cell bodies.[8] The outside of the cerebral hemispheres consists of gray matter called the cerebral cortex, while the inside of the cerebral hemispheres consists of white matter called the center of the medulla.[6] White matter contains myelinated and unmyelinated fibers, oligodendrocytes, fibrous astrocytes, and microglial cells. Gray matter contains perikaryon or nerve cell bodies, unmyelinated fibers, myelinated fibers, astrocytes, oligodendrocytes, and microglial cells. The distinctive color of the white matter indicates the number of myelinated nerve fibers.[7]

The cerebral cortex acts as a center for learning, memory, sensory integration, information analysis, and initiation of motor responses. This structure is composed of gray matter and is estimated to contain 10 billion nerve cells.[9] The most abundant nerve cells are efferent pyramidal cells and are easily observed microscopically.[10]

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.[11, 12] 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.[11] So far, it is not known how filtered kretek cigarette smoke affects the biometrics of brain cells.

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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. 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 and cerebellar, are vulnerable to exposure to foreign substances.[13, 14] Cigarette smoke mostly contains alkaloids. The results of previous studies have shown that cigarette smoke is associated with decreased cognitive function and causes dementia. In more detail, it is stated that cigarette smoke causes thickening of the cortex in the brain.[15] Another study has demonstrated that cigarette smoke inhibits mitochondrial respiration in the rat brain.[16]

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.[17, 18]

Methods

Research design

This study used *Rattus novergicus*, 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. There are 2 groups in this study, namely the treatment group and the control group so the design used is 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) 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.

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Selecting and grouping of experimental animals

Rattus novergicus, Sprague-Dawley strain, aged about 2-3 months with a body weight of 200-250 grams as the inclusion criteria, while the exclusion criteria were if the rats died during treatment. The number of samples is calculated based on the provisions of WHO which states that in experimental research at least 5 animals are used.In this study, 6 rats were used in each group. Group 1 is the control group, while group 2 is the treatment group.

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Treatment of experimental animals

At the beginning of the study, rats were acclimatized for 1 week. Eating and drinking were given to rats during acclimatization. During acclimatization, rats were not treated.After acclimatization, the groups were randomly assigned. Group 1 (6 rats) is the control group, the rats breathe using ordinary air. Group 2 (6 rats) is a group of rats exposed to filtered kretek cigarette smoke 1 stick/day for 3 months (90 days) of treatment.

Filtered kretek cigarette smoke exposure was carried out in a smoking chamber measuring 45 x 35 x 20 cm (31 500 cm^3). The oxygen valve is opened, then the cigarette is attached to the pipe connected to the pump, then the cigarette is burned and the pump is 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^3=27025.740 cm^3). 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 type of feed given is standard feed.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 are provided ad libitum. Feed is given as much as 10% body weight (± 10-15 grams head/day). Feed is given every day in the morning and evening. Drinking water for rats is always changed every day.

After treatment, the rats were anesthetized until they died. Anesthesia using ketamine 100 mg/kg body weight and xylazine 10 mg/kg body weight intra peritoneally. 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 buffer formalin (NBF).

Observation of rat cerebral and cerebellar tissues

The thickness of the cerebral tissue, as well as the cerebellar prepared for making slides, is 3 mm. Rat cerebral tissue, as well as rat cerebellar tissue in paraffin blocks were cut with a thickness of 5 µm. Next, the tissue sections were stained with hematoxylin & eosin (HE). Observations were made on pyramidal cells in the cerebral cortex, i ~~i~~n the hippocampus area. In addition, Purkinje cells were also observed in the gray matter of cerebellar cortex. Observation of ~~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 1and the group 2. Differences between groups were stated 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, are presented in Figure 1.

Figure 1. Brain morphology of Sprague--Dawley rats and sites of tissue sectioning for preparation of slides from the cerebral and cerebellar. Red circle=bregma; white

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circle=lambda; black line=tissue cutting site for making cerebral slides; red line=tissue cutting site for making cerebellar slides.

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.

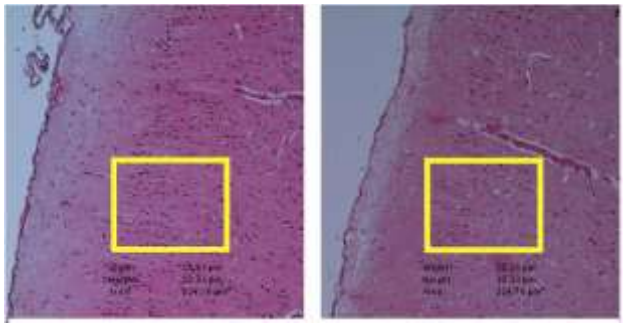

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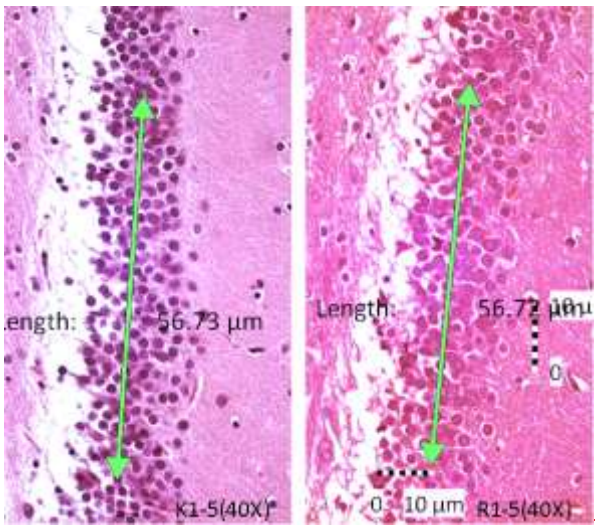
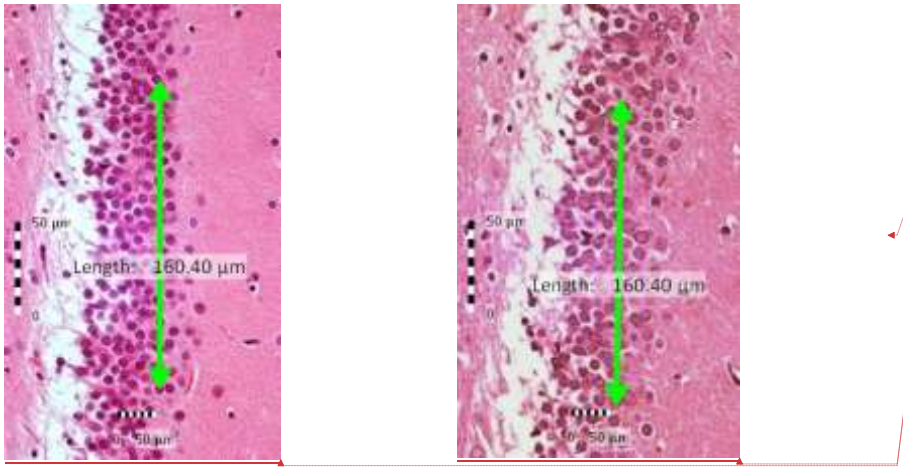
Figure 2. Photomicrograph of rat cerebral cortex, stained with hematoxylin & eosin (objective 10 X). Yellow rectangles are used to show the density of pyramidal cells. A. Cortex cerebral of rat in group 1. B. Cortex cerebral of rat in group 2.





Group 1 Group 2

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

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 in the group 2 showed a decrease in pyramidal cell density.Comparison of pyramidal cell indices in the rats cerebral cortex at the hippocampus area between group 1 and group 2 is presented in Figure 4.

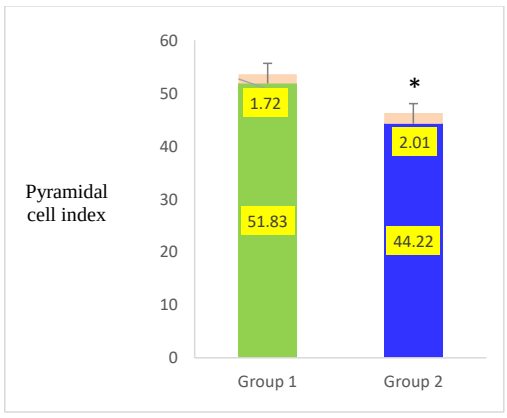


Figure 4. Comparison of pyramidal cell index in the hippocampus area of the rat cerebral cortex. *=significant difference (p<0.001).

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The density of pyramidal cells in the rats cerebral cortex at the hippocampus area between the group 1compared 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 is presented in Figure 5, and Figure 6.

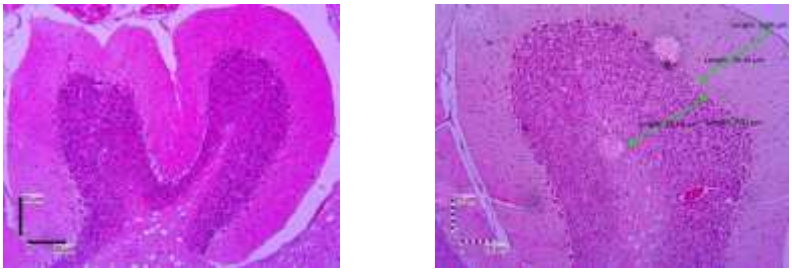

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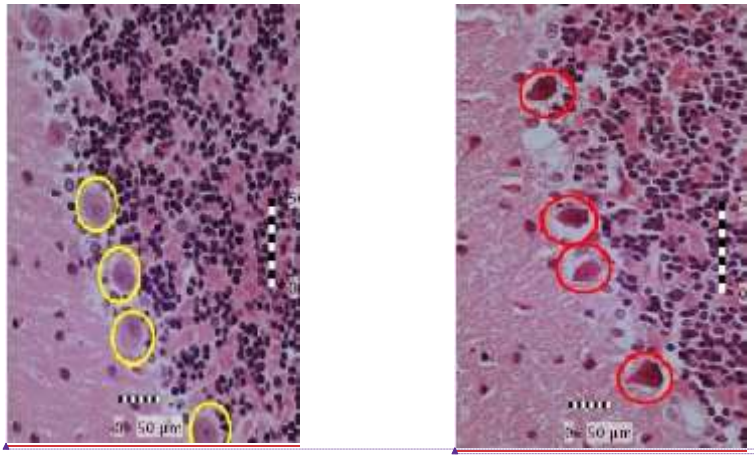
Figure 5. Photomicrograph of rats cerebellar. A. Photomicrograph of rat cerebellar, stained with hematoxylin & eosin, objective 4 X. B. Photomicrograph of rat cerebellar,stained with hematoxylin &eosin, objective 10 X.



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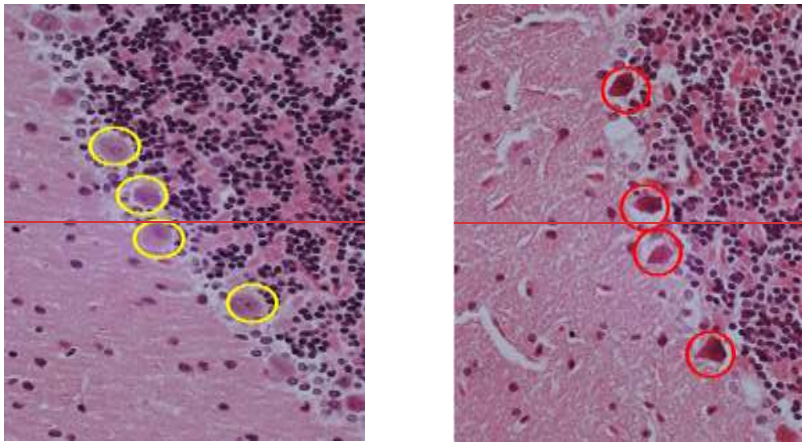

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Figure 6. Histological appearance of the rat cerebellar cortex. A. Rats cerebellar cortex in the group 1, stained with hematoxylin &eosin, objective 40 X. B. Rats cerebellar cortex in the group 2, stained with hematoxylin &eosin, objective 40 X. Purkinje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

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Purkinje cells in the rats cerebellar in the group 1 looks normal, while in the group 2 there was a picture of degenerated.Comparison of Purkinje cell distances in the rat cerebellar between the group 1compared to the group 2 is presented in Figure 7.

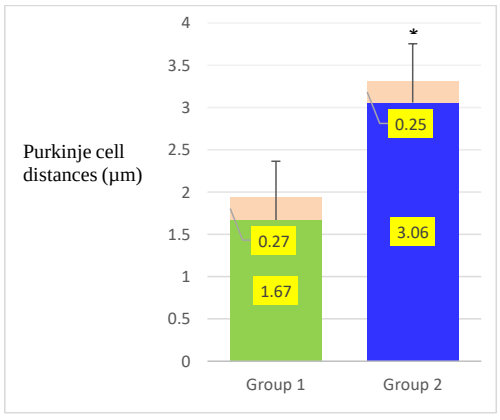

Figure 7. Comparison of Purkinje cell distances in rat cerebellar. *=significant difference (p<0.001).

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Based on Figure 6, the distances of Purkinje cells in the rats cerebellar 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 1compared 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

Tobacco contains alkaloids. Cigarettes sold in the market contain alkaloids about 1.5% by weight of tobacco. Tobacco as the main component of cigarettes accounts for 95% of the total alkaloid content of cigarettes.[19] Nicotine inhaled while smoking takes a short time to reach the brain. The results of a previous study showed that 2 mg of nicotine was absorbed systemically after smoking for 20 minutes by doing 80 puffs on a nicotine inhaler device.[20] Exposure to filtered kretek cigarette smoke in this study can affect the structure and function of the brain.The method of exposure to cigarette smoke in our study is similar to previous studies.[21] Indeed, it is very difficult to arrange so that each rat gets exposure to cigarette smoke in the same amount. To overcome this, it can be done by repairing the smoking box.

The results of photomicroscopy of the cerebral cortex from the frontal lobes of rats showed the outermost part, namely the pia mater which encloses the molecular layer (molecular layer), then external granular, external pyramidal, internal granular, internal pyramidal and the innermost layer is polymorphic. The number of pyramidal cells in the external granular layer at hippocampus area of the group 1 was denser than the group 2. We determined the pyramidal cell density or pyramidal cell density index. This is done by dividing the number of pyramidal cells by a certain line length that passes through the external granular layer. The results of the same study showed that the control group had normal histology in the cerebral cortex layer, while the tramadol treatment showed degeneration of pyramidal cells. Moreover, granular cells characterized by pyknotic nuclei and the appearance of haloes.[22, 23] The results of previous studies showed that tramadol administration caused histological abnormalities and apoptosis in the cerebral cortex which was associated with oxidative stress.[24] In more detail, it is explained that the occurrence of apoptosis is preceded by damage to the DNA structure.[25]

Pyramidal cells in the rat cerebral cortex are easily recognized by the characteristics of the cells being quite large and in large numbers. In the 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 the group 2 compared than the group 1. The results of previous studies demonstrated that pyramidal cell death can be caused by hypoxia,[26, 27] so that the cells experience cell hemostatic disorders.[28]

The cerebellar contains Purkinje cells, located in the ganglion layer. Purkinje cells in the ganglion layer of the rats cerebellar in the group 1 appeared to be round pyramidal in shape. Our results showed that Purkinje cells in the ganglion layer of the rats cerebellar in the group 2 were degenerated and necrotic. Moreover, that the vascularization is getting more and more dense. We suspect this is due to compensatory degeneration and necrosis with increased anastomotic perfusion. In the results of this study, the location of Purkinje cells in the ganglion layer of the rats cerebellar in both the group 1and the group 2 did not show any shift in location. This means that the location of the Purkinje cells in the ganglion layer is still in the right place. Changes in the shape of Purkinje cells in the ganglion layer of the rats cerebellar in the group 2 were clearly pyramidal in shape as a sign of degeneration and necrosis. It is generally stated that cerebellar cognitive function involves interactions between the cerebellar and association areas in the cerebral cortex.[29]

The cerebellar 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.[30] Our results differ from previous studies showing that nicotine at low doses has been shown to be protective in the cerebellar. Administration of nicotine for seven days resulted in mild hyperplasia compared with experimental rats exposed to nicotine for 21 and 42 days. In addition, it was stated that exposure to low doses of nicotine and a short period of time increased the proliferation of neuronal cells.[31]

Our results are in line with previous studies were reported that nicotine causes neurodegeneration in the cerebellar. This is due to the administration of higher doses of nicotine and a longer administration time.[32] Our results are also in agreement with previous studies which demonstrated that Purkinje cells undergo neurodegeneration due to exposure to cigarette smoke in rats.[33] It should be noted from the results of previous studies that low doses of nicotine have a stimulatory effect on the central nervous system.[34] The results of

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our study further strengthen the facts about the dangers of low-dose filtered kretek cigarette smoke in the brains of Sprague--Dawley rats.

Although 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 the cerebral cortex of rats appear abnormal and have decreased in density. In addition, it was also demonstrated that Purkinje cells degenerate and their cell density decreases in the rat cerebellar. In addition, we have not performed immunohistochemical analysis as a specific marker of various cytotypes in both the rat cerebral and cerebellar.

Conclusion

Based on the results of the study, it was found that rats exposed to filtered kretek cigarette smoke 1 stick/day for 3 months decreased the density of pyramidal cells in the ~~hh~~~~ippocampus area of the~~ cerebral cortex at the hippocampus area. The same thing happened that exposure to filtered kretek cigarette smoke 1 stick/day for 3 months decreased Purkinje cell density in the ~~cortex~~ cerebellar cortex.

Acknowledgements

We express our deep gratitude to the Leaders and Staff of the RSHP Laboratory of the Faculty of Veterinary Medicine, IPB-University, Bogor, Indonesia for facilitating in this study.

Conflicts of interest

The authors declare that they have no competing interests.

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Authors 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, and HJE. Approval of the final version of the manuscript: all authors.

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Ethics approval and consent to participate

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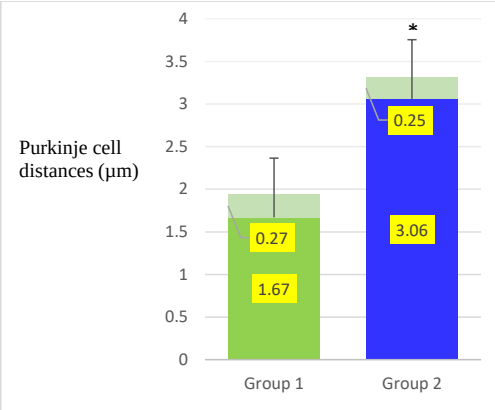

Figure 7. Comparison of Purkinje cell distances in rat cerebellar cortex. *=significant difference ($p<0.001$).

Commented [W1]: The decimals with commas must be corrected to dots in the numbers of y axis

BUKTI REVIEW

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

The work entitled "Decreased density of pyramidal cells in the cerebral cortex, and Purkinje cells in the cerebellar cortex of Sprague Dawley rats after being exposed to cigarette smoke" focuses on a morphological-comparative analysis on the brain of rats exposed to cigarette smoke, with particular emphasis and attention on the pyramidal cells of the cerebral cortex. The work, although interesting overall, has several critical issues that I am going to report below.

It is not clear how the group came to establish the dosage and duration of exposure to cigarette smoke in the group of rats treated with the same, the authors should clarify this point better. It is clear from the discussion that the work contrasts with what was previously shown by Jalili S. et al., in which it is highlighted how low nicotine dosages are (contrary to what the authors stated) associated with an increase in the rate of neuronal proliferation. Therefore, there is a rationale for the dosage and modalities chosen at this point of the discussion (line 250-255), but no previous explicit reference.

There are no references in the text regarding the number of operators who analyzed the histological preparations, how many fields were analyzed and how therefore a statistically reliable curve was obtained.

Furthermore, the work would certainly have benefited if the authors had introduced some immunohistochemical analysis aimed at highlighting specific markers of the various cytotypes examined, other than HE staining.

Few lines need extensive English revisions (i.e., line 46-47)

I suggest to better refine the structure of the manuscript and to better explain the rationale and the methodological approach chosen to pursue the experimental hypothesis.

JBR - Journal of Biological Research [paper #10757] - Editor Decision - Resubmit
2022-09-23 03:46 PM

Dear Drs. EDY PARWANTO, David Tjahyadi, Husnun Amalia, Hosea Jaya Edy,

Your paper entitled "Original Article Decreased density of pyramidal cells in the cerebral cortex, and Purkinje cells in the cerebellar cortex of Sprague Dawley rats after being exposed to cigarette smoke: -" has been examined by our external reviewers and then re-evaluated inhouse.

Peer reviewers found merit in this paper but raised major, constructive criticisms and do not consider this manuscript acceptable for publication in its current form. The reviewers have raised a number of points, listed on the web site and provided below/attached for your convenience.

The editorial conclusion is that substantial changes should be made to meet the reviewers' criticisms.

Your revised manuscript should be accompanied by a covering letter to explain, point by point, how you have modified your paper in answer to the reviewer's comments.

Important: we recommend that you consult the Authors' guidelines of this journal under Submission, as well as its current contents, to ensure that your revised manuscript is written in accordance to the journal editorial standards (in particular, title page, tables and references style).

The revised manuscript, edited in .DOC format, should be resubmitted electronically within 3 weeks from the date of the Editor Decision message.

In order to resubmit the revised manuscript, please follow this step-by-step procedure:

i) log into the journal using your username and your password;
ii) click on your role as ‘Author’;
iii) click on the correct title;
iv) click on ‘Review’ on the page displayed;
v) under the heading ‘Editor decision’ (bottom-page), upload the revised paper. Use ‘Browse’ to find the files and ‘Upload’ to upload them;

vi) once the files are uploaded, inform the Managing Editor and the Editor-in-Chief of the Journal via e-mail.
Following this procedure, you can upload one or more files (max 8 MB each file). Make sure to click ‘Upload’ for each single file you would like to upload.

Moreover, although we encourage resubmission, please be aware that this is not a statement of acceptance or a promise to accept a revised manuscript. The final decision as to this paper's acceptability for publication will exclusively depend on how our current concerns are met.

Thank you very much for sending this work to our journal: we look forward to receiving a revised manuscript.

With kind regards,

Dr. Gian Luigi Mariottini, MSc, MD, Editor in Chief,
University of Genova (retired), Research Fellow Department of Earth, Environment and Life Sciences, Genova, Italy. Gian.Luigi.Mariottini@unige.it
glmariottini@libero.it

Prof. Filippo Macaluso, Associate Editor, Telematic University eCampus, Italy.
filippo.macaluso1@uniecampus.it

Reviewer A:

The study of Tjahyadi and colleagues aims to determine the effect of kretek cigarette smoke on the density of pyramid cells in the cerebrum, and Purkinje cells in the cerebellum of mice.

The paper, although not complitely original and innovative, is putatively interesting for this Journal but they have to modify some figures before we can accept in for publication:

1. In figure 2, the microphotographs have probably a different light exposition or contrast, so that in A) the nuclei are in blue (as normal for the H&E staining) but in B) the look in pink. This fact does not permit a correct evaluation of the difference. The authors should present representative microphotographs with the same contrast/colour (i.e., nuclei in violet/blue) as per normal H&E stainings. See for example figure 5 where the staining appears with the same colours.

2. Figure 3 and 6 are histograms. They should show a difference between groups. However, the high of the column are the same (althouh the number above them are different). This is an error that the authors should correct.

Lastly, the authors exposed group 2 (6 rats) to cigarette smoke 1 stick/day for 3 months (90 days) of treatment. How many years of life in humans is 3 months in the rat? Authors should add this information, with appropriate bibliographic references, to their work.

Recommendation: Major Revisions

Reviewer M:

The manuscript entitled “Decreased density of pyramidal cells in the cerebral cortex, and Purkinje cells in the cerebellar cortex of Sprague Dawley rats after being exposed to cigarette smoke” provides evidence on structural modifications encountered in a rat model of Kretek cigarette smoke at cerebral and cerebellar level. This paper necessitates a methodological and stylistic revision before being ready for publication in the Journal of Biological Research. Generally speaking, the Authors should better substantiate data provided with adequate statistical information and methodological explanations. Furthermore, an in-depth revision of the text by a Native Speaker is not avoidable. In particular, the following are the main points to take into account:

- The introduction extensively reports a basic anatomical description of the areas investigated that could appear redundant. I suggest to better targeting the introduction on the innovative aspects brought about by this study, i.e. the Kretek cigarettes effects. What peculiarities do they have? What are the other studies already present in the field that can substantiate this experimental choice? Maybe adding a chemical composition of the Kretek cigarettes would help too.
- The title should be modified to enhance the originality of the study. Instead of a general “cigarette smoke” it would be better to refer to Kretek smoke.

- Methodologically, I have concerns. Firstly, I have strong doubts that the exposure system described by the Authors could allow proper control of the passive smoke. Have they ever monitored the CO2 amount inside the chamber? Secondly, the authors should better substantiate the treatment choice. Is it sufficient to only administer 1 stick (10 minutes) per day? Is there any reference for the protocol chosen? For instance, in this paper https://doi.org/10.1016/j.lfs.2020.117644 it is 1 hour for 5-6 cigarettes in order to monitor the effect. Lastly, it would be important to better detail how the cell counting has been performed. In the methods section, it is just indicated that “observations" were made. Definitely, this part should be better explained and justified.

- Regarding the results, I noticed that no statistical values have been provided in the text apart from the p values. Since the authors used a Student T-test, they should at least report the degree of freedom (df) and t values. Also, the graphs do not seem consistent with the mean and SD values reported over them. I would ask you to explain the reason for this discrepancy.
- The authors asserted they have explored the density of hippocampus pyramidal cells. What was the exact localization investigated?

- The abstract should be re-written because it does not contain a structured distribution into background, aim, methods, results and conclusions.
- Last but not least, I believe that the manuscript requires a powerful revision by a Native speaker for improving the overall quality of language.

Recommendation: Major Revisions

ORIGINAL ARTICLE

Decreased density of pyramidal cells in the cerebral cortex, and Purkinje cells in the cerebellar cortex of Sprague-Dawleyrats after being exposed to filtered kretekcigarette smoke

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Abstract

Filtered kretek cigarette smoke is in the form of gas containing solid materials (particulates). Such cigarette smoke contains carcinogenic substances. In addition, it is stated that tobacco has a significant negative impact on the development of neural structures, causes addiction, and affects brain activity and function.

Aimof this paper wasto determine the effect of filtered kretek cigarette smoke on the density of pyramidal cells in the cerebral cortex, and Purkinje cells in thecerebellar cortex.

The study was conducted experimental and control group design. Group 1 (6 rats) is the control group, the rats breathe using ordinary air. Group 2 (6 rats) is a group of rats exposed to filtered kretek cigarette smoke 1 stick/day for 3 months of treatment.Observations were made on pyramidal cells in the rat cerebral cortex, and Purkinje cells in the cerebellar cortex.

The distribution of pyramidal cells in the cerebral cortex at the hippocampus area of rats in the group 1 was expected. In contrast, the group 2 showed a decrease in the density of pyramidal cells.There was a difference in the density of pyramidal cells in the rat cerebral cortex between the group 1 compared to the group 2 ($p<0.001$).Purkinje cells in the cerebellar cortexin group 1 were normal, while in group 2 there was a picture of degenerated Purkinje cells. The distance between Purkinje cells in the cerebellar cortexof the group 1 was denser compared to the group 2 ($p<0.001$).

Rats exposed to kretek cigarette smoke 1 stick/day for 3 months decreased pyramidal cell density in the cerebral cortexat the hippocampus area. The same thing happened: exposure to kretek cigarette smoke 1 stick/day for 3 months decreased Purkinje cell density in the cerebellar cortex.

Keywords: cerebral cortex, cerebellar cortex,filtered kretek cigarette smoke, pyramidal cell, cerebellarPurkinje cell.

Introduction

Smoking is a global problem that causes health problems. Around 2.5 billion people in the world are smokers and that number is in third-world countries. Indonesia's population is included in the group of third-world countries, meaning that many of the population consume cigarettes.[1]

Cigarette smoke is a gas that contains solid materials (particulates). In addition, cigarette smoke is also known to contain carcinogenic ingredients. Moreover, it is stated that tobacco has a significant negative impact on the development of nervous structures,[2]and cause addiction.[3]Gases and particulates of cigarettes are channeled into the alveoli.[4]In addition, it is also stated that smoking affects the activity and function of the brain and interferes with the psychology of cigarette addicts.[5]

The cerebralcortex is the largest and most prominent part of the brain; it is divided into the right and left hemispheres by a deep groove or fissure called the major longitudinal fissure.[6]The surface of the cerebralcortex is extended by many gyri, which are elevations and are separated by grooves called sulci.[7]The cerebral cortex has neurons with pyramid-shaped cell bodies.[8]The outside of the cerebral hemispheres consists of gray matter called the cerebral cortex, while the inside of the cerebral hemispheres consists of white matter called the center of the medulla.[6]White matter contains myelinated and unmyelinated fibers, oligodendrocytes, fibrous astrocytes, and microglial cells. Gray matter contains perikaryon or nerve cell bodies, unmyelinated fibers, myelinated fibers, astrocytes, oligodendrocytes, and microglial cells.The distinctive color of the white matter indicates the number of myelinated nerve fibers.[7]

The cerebral cortex acts as a center for learning, memory, sensory integration, information analysis, and initiation of motor responses. This structure is composed of gray matter and is estimated to contain 10 billion nerve cells.[9]The most abundant nerve cells are efferent pyramidal cells and are easily observed microscopically.[10]

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.[11, 12]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.[11]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.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 cerebralcortex and ~~cerebellar~~cerebellum, are vulnerable to exposure to foreign substances.[13, 14]Cigarette smoke mostly contains alkaloids. The results of previous studies have shown that cigarette smoke is associated with decreased cognitive function and causes dementia. In more detail, it is stated that cigarette smoke causes thickening of the cortex in the brain.[15]Another study has demonstrated that cigarette smoke inhibits mitochondrial respiration in the rat brain.[16]

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-Dawleyrats 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.[17, 18]

Materials and methods

Research design

This study used *Rattus novergicus*, Sprague-Dawleystrain, to test the effect of filtered kretek cigarette smoke on pyramidal cells in the cerebral cortex and Purkinje cell density in the cerebellar cortex.There are 2 groups in this study, namely the treatment group and the control group so the design used is 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, UniversitasTrisakti, 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, UniversitasTrisakti, with No: 184/KER/FK/VIII/2018.

Selecting and grouping of experimental animals

Rattus novergicus, Sprague-Dawley strain, aged about 2-3 months with a body weight of 200-250 grams as the inclusion criteria, while the exclusion criteria were if the rats died during treatment.The number of samples is calculated based on the provisions of WHO which states that in experimental research at least 5 animals are used.In this study, 6 rats were used in each group. Group 1 is the control group, while group 2 is the treatment group.

Treatment of experimental animals

At the beginning of the study, rats were acclimatized for 1 week.Eating and drinking were given to rats during acclimatization. During acclimatization, rats were not treated.After acclimatization, the groups were randomly assigned.Group 1 (6 rats) is the control group, the rats breathe using ordinary air.Group 2 (6 rats) is a group of rats exposed to filtered kretek cigarette smoke 1 stick/day for 3 months (90 days) of treatment.

Filtered kretek cigarette smoke exposure was carried out in a smoking chamber measuring 45 x 35 x 20 cm (31 500 cm^3). The oxygen valve is opened, then the cigarette is attached to the pipe connected to the pump, then the cigarette is burned and the pump is 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^3=27025.740 cm^3).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 type of feed given is standard feed.~~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 wereprovided ad libitum.Feed wasgiven as much as 10% body weight (± 10-15 grams head/day)~~.Feed is given~~ , every day in the morning and evening. Drinking water for rats wasalways changed every day.

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After treatment, the rats were anesthetized until they died.Anesthesia using ketamine 100 mg/kg body weight and xylazine 10 mg/kg body weight intra peritoneally.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 buffer formalin (NBF).

Observation of rat cerebral and cerebellar tissues

The thickness of the cerebral tissue, as well as the cerebellar prepared for making slides, is 3 mm.Rat cerebral tissue, as well as rat cerebellar tissue in paraffin blocks were cut with a thickness of 5 µm.Next, the tissue sections were stained with hematoxylin andeosin (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 1and the group 2.Differences between groups were stated if the results of the analysis showed a p value < 0.05.

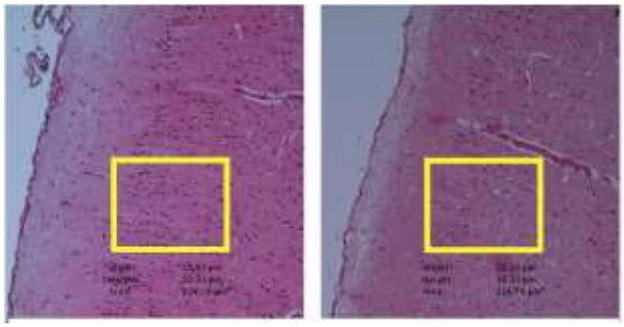
Results

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.

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

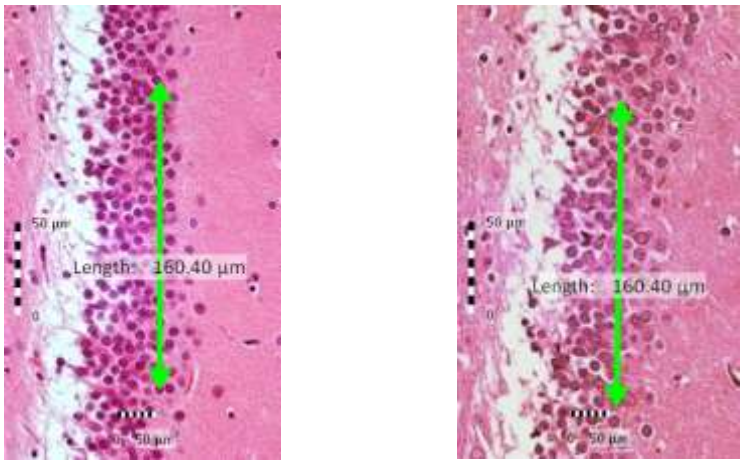
circle=lambda; black line=tissue cutting site for making cerebral slides; red line=tissue cutting site for making cerebellar slides.

Photomicrograph of rat cerebral cortexin 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.



A. B.

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



Group 1 Group 2

Figure 3. Hippocampus area of cerebral cortex ~~cerebral~~ in rat. A. Hippocampus area of cerebral cortex ~~cerebral~~ in group 1, stained with hematoxylin andeosin (objective 40 X). B.

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Hippocampus area of cerebral cortex ~~cerebral~~ in group 2, stained with hematoxylin and eosin (objective 40 X).

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 in the group 2 showed a decrease in pyramidal cell density.Comparison of pyramidal cell indices in the rats cerebral cortex at the hippocampus area between group 1 and group 2 is presented in Figure 4.

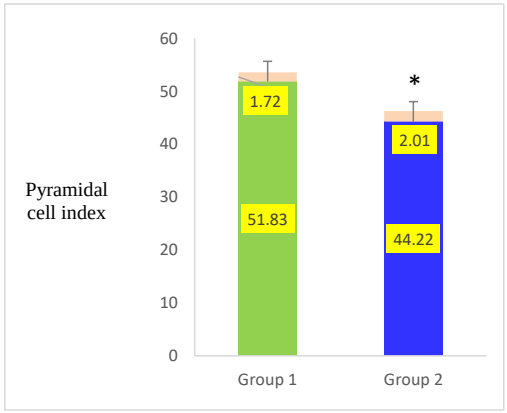
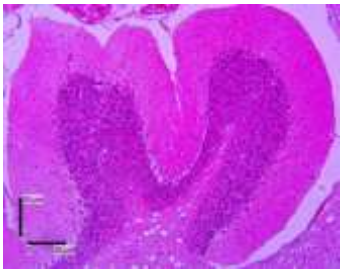


Figure 4. Comparison of pyramidal cell index in the hippocampus area of the rat cerebral cortex. *=significant difference (p<0.001).

The density of pyramidal cells in the rats cerebral cortex at the hippocampus area between the group 1compared 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.



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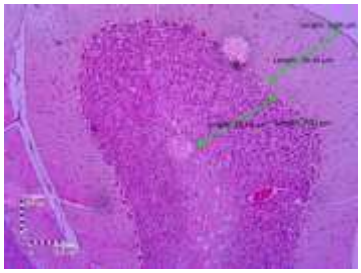

B.

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

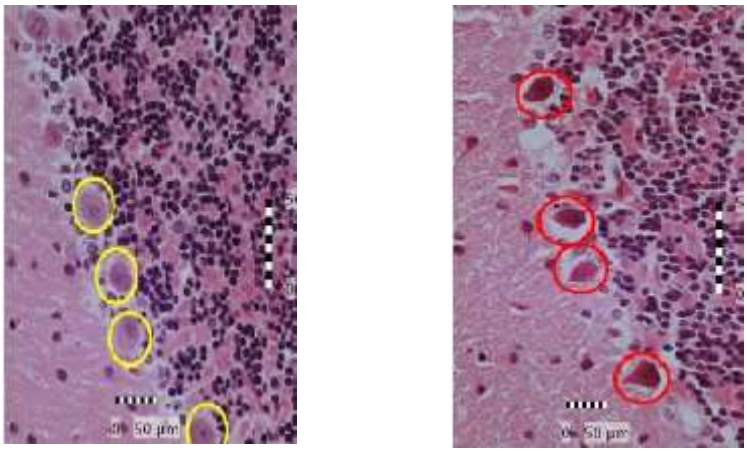

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Figure 6. Histological appearance of the ratcerebellar cortex. A. Rats cerebellar cortex in the group 1, stained with hematoxylin and eosin, objective 40 X. B. Rats cerebellar cortex in the group 2, stained with hematoxylin and eosin, objective 40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

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Purkinje cells in the rats cerebellar cortexin the group 1 looks normal, while in the group 2 there was a picture of degenerated.Comparison of Purkinje cell distances in the rat cerebellar cortex between the group 1compared to the group 2 is presented in Figure 7.

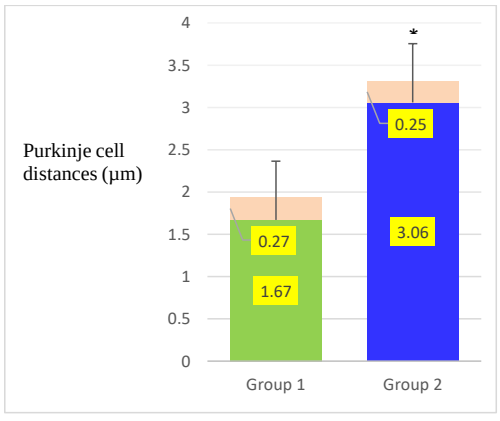

Figure 7. Comparison of Purkinje cell distances in rat cerebellar cortex. *=significant difference (p<0.001).

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Based on Figure ~~6~~7, the distances of Purkinje cells in the rats cerebellar cortexof 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 1compared 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

Tobacco contains alkaloids. Cigarettes sold in the market contain alkaloids about 1.5% by weight of tobacco. Tobacco as the main component of cigarettes accounts for 95% of the total alkaloid content of cigarettes.[19]Nicotine inhaled while smoking takes a short time to reach the brain. The results of a previous study showed that 2 mg of nicotine was absorbed systemically after smoking for 20 minutes by doing 80 puffs on a nicotine inhaler device.[20]Exposure to filtered kretek cigarette smoke in this study can affect the structure and function of the brain.The method of exposure to cigarette smoke in our study is similar to previous studies.[21]Indeed, it is very difficult to arrange so that each rat gets exposure to cigarette smoke in the same amount. To overcome this, it can be done by repairing the smoking box.

The results of photomicroscopy of the cerebral cortex from the frontal lobes of rats showed the outermost part, namely the pia mater which encloses the molecular layer (molecular layer), then external granular, external pyramidal, internal granular, internal pyramidal and the innermost layer is polymorphic.The number of pyramidal cells in the external granular layer at hippocampus area of the group 1 was denser than the group 2.We determined the pyramidal cell density or pyramidal cell density index. This is done by dividing the number of pyramidal cells by a certain line length that passes through the external granular layer.The results of the same study showed that the control group had normal histology in the cerebral cortex layer, while the tramadol treatment showed degeneration of pyramidal cells. Moreover, granular cells characterized by pyknotic nuclei and the appearance of haloes.[22, 23]The results of previous studies showed that tramadol administration caused histological abnormalities and apoptosis in the cerebral cortex which was associated with oxidative stress.[24]In more detail, it is explained that the occurrence of apoptosis is preceded by damage to the DNA structure.[25]

Pyramidal cells in the rat cerebral cortex are easily recognized by the characteristics of the cells being quite large and in large numbers.In the 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 the group 2 compared than the group 1.The results of previous studies demonstrated that pyramidal cell death can be caused by hypoxia,[26, 27]so that the cells experience cell hemostatic disorders.[28]

The cerebellarcortex contains Purkinje cells, located in the ganglion layer.Purkinje cells in the ganglion layer of the rats cerebellarcortex in the group 1 appeared to be round pyramidal in shape.Our results showed that Purkinje cells in the ganglion layer of the rats cerebellarcortex in the group 2 were degenerated and necrotic.Moreover, that the vascularization is getting more and more dense. We suspect this is due to compensatory degeneration and necrosis with increased anastomotic perfusion.In the results of this study, the location of Purkinje cells in the ganglion layer of the rats cerebellarcortex in both the group 1and the group 2 did not show any shift in location. This means that the location of the Purkinje cells in the ganglion layer is still in the right place.Changes in the shape of Purkinje cells in the ganglion layer of the rats cerebellarcortex in the group 2 were clearly pyramidal in shape as a sign of degeneration and necrosis.It is generally stated that cerebellar cognitive function involves interactions between the cerebellarcortex and association areas in the cerebral cortex.[29]

The cerebellarcortex 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.[30]Our results differ from previous studies showing that nicotine at low doses has been shown to be protective in the cerebellar cortex. Administration of nicotine for seven days resulted in mild hyperplasia compared with experimental rats exposed to nicotine for 21 and 42 days.In addition, it was stated that exposure to low doses of nicotine and a short period of time increased the proliferation of neuronal cells.[31]

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.[32]Our results are also in agreement with previous studies which demonstrated that Purkinje cells undergo neurodegeneration due to

exposure to cigarette smoke in rats.[33]It should be noted from the results of previous studies that low doses of nicotine have a stimulatory effect on the central nervous system.[34]The results of our study further strengthen the facts about the dangers of low-dose filtered kretek cigarette smoke in the brains of Sprague-Dawley rats.

Although 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 the cerebral cortex of rats appear abnormal and have decreased in density. In addition, it was also demonstrated that Purkinje cells degenerate and their cell density decreases in the rat cerebellar cortex. In addition, we have not performed immunohistochemical analysis as a specific marker of various cytotypes in both the rat cerebral and cerebellar cortex.

Conclusion

Based on the results of the study, it was found that rats exposed to filtered kretek cigarette smoke 1 stick/day for 3 months decreased the density of pyramidal cells in the cerebral cortexat the hippocampus area. The same thing happened that exposure to filtered kretek cigarette smoke 1 stick/day for 3 months decreased Purkinje cell density in the cerebellarcortex.

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Conflicts of interest

The authors declare that they have no competing interests.

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Authors 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, and HJE. Approval of the final version of the manuscript: all authors.

Ethics approval and consent to participate

Not applicable.

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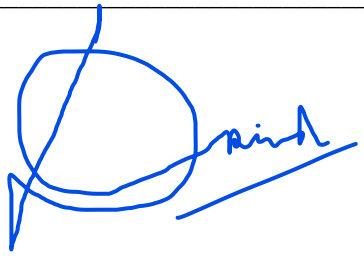

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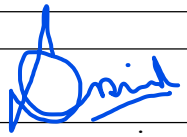 — your signature

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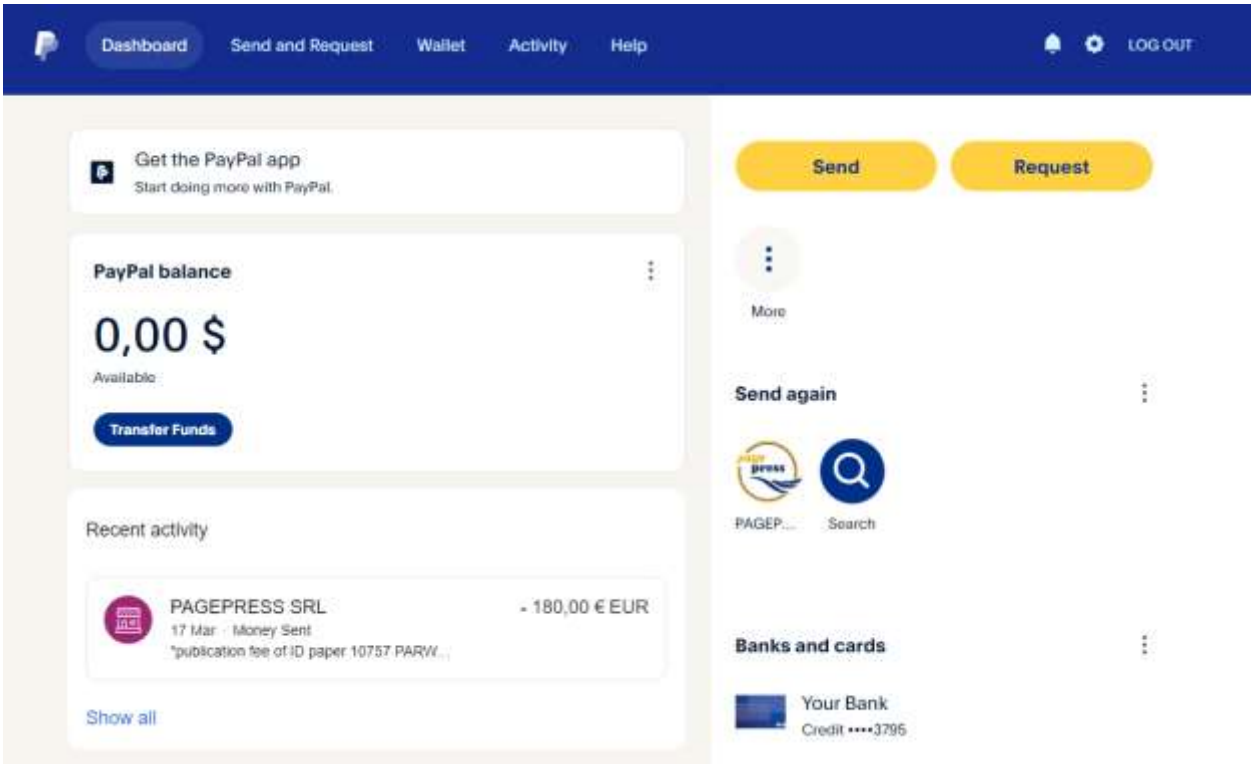
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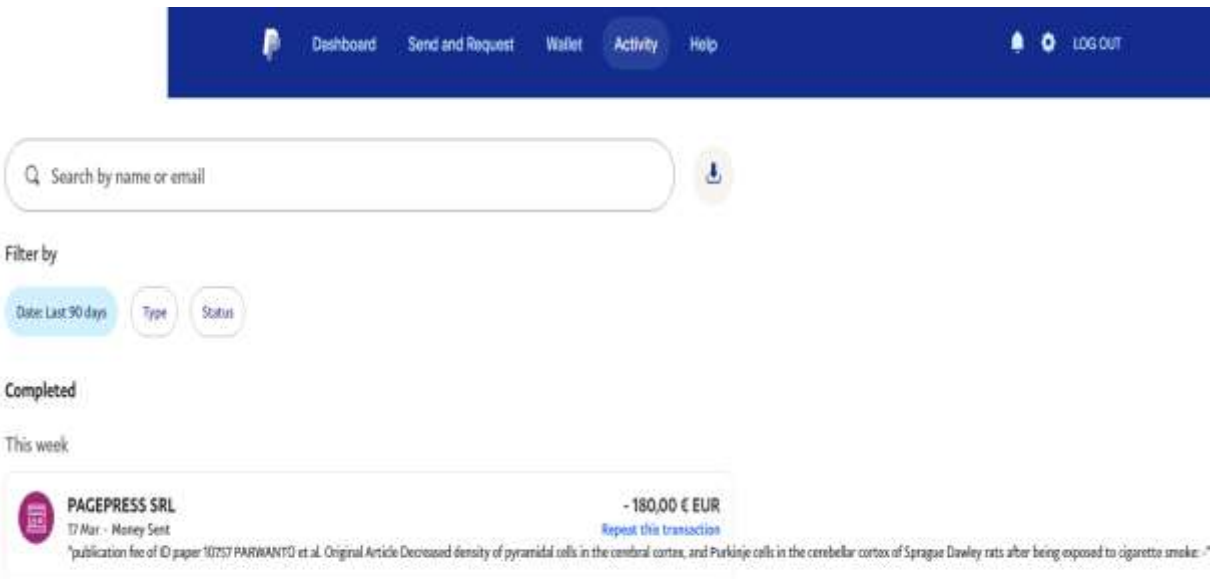
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