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*RESEARCH ARTICLE*

# Histometric Analysis to Perform Purkinje cell Abnormalities in Rats Due to Low doses of Kretek Smoke Exposure

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## ABSTRACT:

Facts show that many Indonesians are smokers. Some of these smokers are kretek smokers. Kretek is made from a mixture of tobacco, cloves, and sauce. Kretek smoke is toxic, and affects the cerebellum, especially Purkinje cells. Aim of this study was to determine the effects of kretek smoke on the Purkinje cells of rats. CG (control group) were rats that breathed using normal air (not exposed to kretek smoke). TG (treatment group) were rats that were exposed to smoke of kretek 1 stick/day for 1 month (30 days). Observations were made on the morphology of the rat brain and histology on the cerebellum. Qualitative and quantitative observations were made on Purkinje cells (area, perimeter, length, and width). The nicotine content in the kretek cigarettes used in this study was 3.88±0.05µg/g. Exposure to kretek smoke of 1 stick/day for 1 month in rats demonstrated that the brain did not experience changes in shape, but Purkinje cells in the cerebellum became abnormal in both shape and size. Purkinje cells area of rats in CG was higher than TG ($p = 0.000$). Purkinje cells perimeter of rats in CG was higher than TG ($p = 0.000$). Purkinje cells length of rats in CG was higher than TG ($p = 0.000$). Purkinje cells width of rats in CG was higher than TG ($p = 0.000$). Exposure to kretek smoke of 1 stick/day for 1 month did not change the morphology of the rat brain, but reduced the size of Purkinje cell bodies, and changed the shape from round to tapered as a sign of cell degeneration.

**KEYWORDS:** Kretek, Low-Dose Kretek Smoke, Nicotine, Cerebellum, Purkinje.

## INTRODUCTION:

Indonesia is a group of countries whose population is a smoker in large numbers.[1] Based on the results of the Basic Health Research survey of the Ministry of Health of the Republic of Indonesia in 1918, it shows that 34% of the Indonesian population aged 15+ years are smokers.[2] Most smokers in Indonesia, namely 73%, smoke kretek.[3] The characteristic of kretek is that its composition consists of tobacco, cloves, and sauces.[4]

Cigarette smoke is a toxic mixture. It is known that more than 7000 chemicals are contained in cigarette smoke, more than 4500 as a toxin, and 70 of them are known to be carcinogenic. Nevertheless, there is no doubt that tobacco has been used as an addictive stimulant for

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people around the world.[5] Moreover, it has been demonstrated that 24 types of kretek cigarettes contained 2.8-33.8mg/stick of eugenol. Fourteen types of 24 types of kretek cigarettes contain 2.8-12.9mg/stick of menthol, 5 of 9 cigarettes contain 3.6-10.8mg/stick.[6] In addition, that tobacco as the main component of kretek contains nicotine. Nicotine that enters the body when smoking will spread quickly to the brain. Previous studies have shown that nicotine is absorbed systemically after smoking.[7] Apart from that, the substances contained in cigarette smoke cause addiction.[8] In this regard, it is stated that the content of this substance influences the development of nerve structures.[5] Smoking also causes motor damage to the brain.[9]

Our previous study which shown that smoke of filtered kretek decreased the size of bronchioles, but increased the size of respiratory bronchioles. It was further demonstrated that filtered kretek smoke also affected to overexpression of the p53 gene.[10] Moreover, also demonstrated that components of tobacco smoke impact gene expression in *E. coli*.[11] Therefore, it is necessary to further investigate the relationship between gene expression manifestations (due to exposure to cigarette smoke) and target cells.

Parts of the brain include the cerebellum. Phylogenetically, the cerebellum is classified into 3 parts, namely paleocerebellum, neocerebellum, and archicerebellum. Based on its anatomical location, the cerebellum is divided as follows: the anterior lobe of the cerebellum, namely the paleocerebellum, the posterior lobe, namely the neocerebellum, and the vestibule of the cerebellum or flocculonodular lobe, namely the archicerebellum. The cerebellum is responsible for motor control. Purkinje cells line up in rows called the Purkinje cell layer. Purkinje cell bodies have unique dendritic branches.[12] The cerebellum can be used as a model for studying the central nervous system. The choice of the cerebellum as a learning model is based on its precise development, so that it can be used as a model for identifying disorders of the central nervous system.[13]

The research results show that in society many people smoke at low doses.[14,15] Because that, we focused on the effects at low doses of kretek smoke on the rat brain. The effects observed in this study were histological of cells abnormalities in the cerebellum. This focus is a continuation of our previous research.[16,17]

Although Purkinje cell density has been proven histometrically, it is necessary to know the smoke effect of kretek on brain cell histometrics.[16] So, the purpose of this research is to determine the effect of kretek smoke on changes in “rat Purkinje cells”. Changes in "rat Purkinje cells" both qualitatively and quantitatively can be used as a guideline in a more objective diagnosis of brain tissue disorders.

## MATERIAL AND METHODS:

***Kretek:***

The kretek used in this research were made manually with a composition of tobacco, cloves, and sauce is called as “woor”. Tobacco, cloves, and sauce were purchased at the market in the Special Region of Yogyakarta, Indonesia.

**Measurement of nicotine levels:**

Measurement of nicotine levels in kretek used the CAMAG Linomat 5 "Linomat5_220727" S/N 220727 (1.00.13)instrument. The test method used is Thin Layer Chromatography (TLC) Densitometry. The weight of the sample (kretek) used was 500 mg. Nicotine standards were dissolved in ethanol. Nicotine spot density is read at a wavelength of 265 nm. The retardation factor (Rf) value in measuring nicotine levels is 0.64, with a standard of 1010 ug/mL. The formula used to calculate nicotine levels is as follows:

$$\text{Nicotin in the sample (ug)} = \frac{(\text{sampel area} - b)}{a}$$

$$\text{LoD device (ug)} = \frac{(v2 * b) - b}{a}$$

$$\text{LoD in sample} = \frac{\text{LoD device (ug)}}{\text{Number of spottings in the sample (ug)}} * 1000$$

$$\text{Sample nicotine levels (ug/g)} = \frac{\text{Nicotine levels in the device (ug)}}{\text{Number of samples posted (ug)}} * 1000$$

**Screening GC MS:**

The GC–MS tool used to measure the substance content in kretek requires first calibrating the tool. The sample (kretek) was added with 1.5 mL of ethanol in a microtube, then vortexed until homogeneous. Next, the solution was centrifuged at 9500 rpm for 5 minutes. The supernatant is put into a gas chromatography vial, then injected into the gas chromatography column. Column used HP-5ms Ultra Inert Columns.

**Ethical clearance:**

We obtained the ethical clearance No. 058/KER/FK/IU2024 from the Research Ethics Commission, Faculty of Medicine, Universitas Trisakti, Jakarta, Indonesia.

**Animal:**

The experimental animals used were the Sprague-Dawley rats. The number of rats is16, male gender, age: 2 – 3 months, body weight 150 to 200grams, in good health as declared by a veterinarian. During the study, rats were placed in cages individually. Rat cages were placed in an animal treatment room. The room temperature used for these experimental was 24 - 28°C, with a room humidity of 50-60%. The room lighting used

was artificial fluorescence with a light and dark cycle of 12:12 hours. The rats were given food and drink sufficiently (at libitum), based to standards.

Sample size calculated with the minimum sample size formula, namely N = 10: (2 – 1) + 1 = 5 rats, while the maximum sample size was N = 20:(2 – 1) + 1=10 rats.[18] Researchers used 8 rats for each treatment group. Rats were sacrificed after the experiment was completed. Brain organs were taken to make slide preparations.

**Treatment:**
Acclimatization for 1 week was carried out on all rats included in the study. During acclimatization, rat was in individual cages placed in the animal treatment room. The rats were given food and drink at libitum, according to standards. During acclimatization, rats were not treated. After acclimatization, the rats were randomly grouped into 2 groups, each group consisting of 8 rats. Control group in these studies is CG. CG is rats breathe normal air, not exposed to smoke of kretek. Treatment group in this study is TG, the rats were given treatment, namely exposure to smoke of kretek, with a dose of 1 cigarette/day. The duration of exposure to smoke of kretek on both groups (CG and TG) was 1 month (30 days).

The size of the glass box for treatment is 45 cm long, 35 cm wide and 20cm high (31,500 $cm^3$). The glass box as a place for cigarette smoke exposure is equipped with a pipe connected to an electric pump. After the kretek is burned, and the electric pump is turned on, the smoke of kretek enters the glass box, then is inhaled by the experimental rats.

Treatment in TG for 10 minutes, every morning for 1 month (30 days). After being treated, the rats were anesthetized to death. We used ketamine (100mg/kg body weight), and xylazine (10mg/kg body weight) intraperitoneally. Animals that had been sacrificed by euthanasia, then their brains were taken, and stored in bottles containing 10% neutral buffered formalin (NBF) solution. The next step is that the brains are used to make histological preparations.

**Research diagram:**
Research diagram is presented in Figure 1.

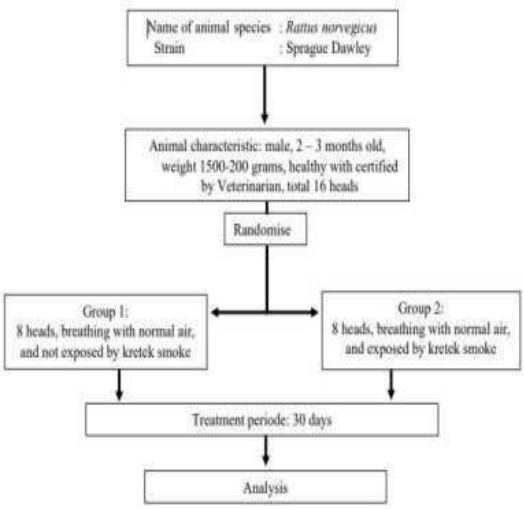


**Figure 1. Research flow diagram**

**Hematoxylin and eosin staining:**
NBF 10% is used to fix the brain tissue. Next, the brain tissue was made into slides with hematoxylin and eosin staining as done in previous studies.[16, 17]

**Qualitative observed of Purkinje cells:**
Cerebellum slide images were documented using Optilab Advance Plus. Analysis of the slide images used ImageRaster 3. Optilab Advance Plus, and Image Raster 3 devices (https://miconos.ac.id/new/support/download). Observation of Purkinje cell abnormalities was shown if abnormalities in cell shape were found. The shape of Purkinje cells between the control group and the treatment group was compared qualitatively.

**Quantitative measurement of Purkinje cells:**
Cerebellum image documentation has been carried out with Optilab Advance Plus. Quantitative measurement of Purkinje cells was carried out with Image Raster 3 (https://miconos.ac.id/new/support/download).
Cytometry parameters of Purkinje cells are area, perimeter, length, and width. Three researchers were involved in the measurement of Purkinje cell cytometry. Measurement data are displayed as mean and standard deviation (SD). Independent sample t test was used to compare differences in Purkinje cell cytometry between groups. We used $p<0.05$ for differences between groups.

## RESULTS:

**Nicotine levels:**
Chromatogram obtained using <win CATS Planar Chromatography Manager= and the standard curve used to measure nicotine levels of kretek are presented in Figure 2.

**Table 1. Nicotine levels in kretek cigarettes**

| NS | R | SW | SSV | AFS | Freq | NDS | A | NIS | NIDS | Avr |
|---|---|---|---|---|---|---|---|---|---|---|
| | | (g) | (µL) | mL | | (µg) | | (µg) | (µg/g) | (µg/g) |
| **1** | 1 | 0.5048 | 2.5 | 5 | 1 | 252.4 | 5916.1 | 0.99 | 3.92 | 3.92 |
| | 2 | 0.5048 | 2.5 | 5 | 1 | 252.4 | 5916.1 | 0.99 | 3.92 | |
| **2** | 1 | 0.5022 | 2.5 | 5 | 1 | 251.1 | 5840.5 | 0.975 | 3.88 | 3.84 |
| | 2 | 0.5022 | 2.5 | 5 | 1 | 251.1 | 5724.9 | 0.951 | 3.79 | |
| **3** | 1 | 0.5034 | 2.5 | 5 | 1 | 251.6 | 5752.6 | 0.957 | 3.80 | 3.80 |
| | 2 | 0.5034 | 2.5 | 5 | 1 | 251.8 | 5759.3 | 0.958 | 3.81 | |
| **4** | 1 | 0.5029 | 2.5 | 5 | 1 | 251.2 | 5838.4 | 0.974 | 3.88 | 3.88 |
| | 2 | 0.5029 | 2.5 | 5 | 1 | 251.2 | 5852.5 | 0.977 | 3.89 | |
| 5 | 1 | 0.5041 | 2.5 | 5 | 1 | 251.9 | 5798.4 | 0.966 | 3.84 | 3.91 |
| | 2 | 0.5041 | 2.5 | 5 | 1 | 251.9 | 5976.3 | 1.002 | 3.98 | |
| 6 | 1 | 0.5046 | 2.5 | 5 | 1 | 252.2 | 5921.2 | 0.991 | 3.93 | 3.93 |
| | 2 | 0.5046 | 2.5 | 5 | 1 | 252.2 | 5928.3 | 0.993 | 3.94 | |

Avr ± SD 3.88 ± 0.05

Abbreviations: NS = Number of Sample, R = Replicants, SW = Sample weight, SSV = Sample spotting volume, AFS = Add final sample, F = Frequency, NDS = Number of deep spots, A = area, NIS = nicotine in the sample, NIDS = Nicotine levels in dry samples, Avr = average, g = gram, µL = micro liter, mL = milli liter, µg = micro gram, µg/g = micro gram per gram, SD = standard of deviation.

The nicotine content in kretek used in this study was 3.88 ± 0.05 µg/g.

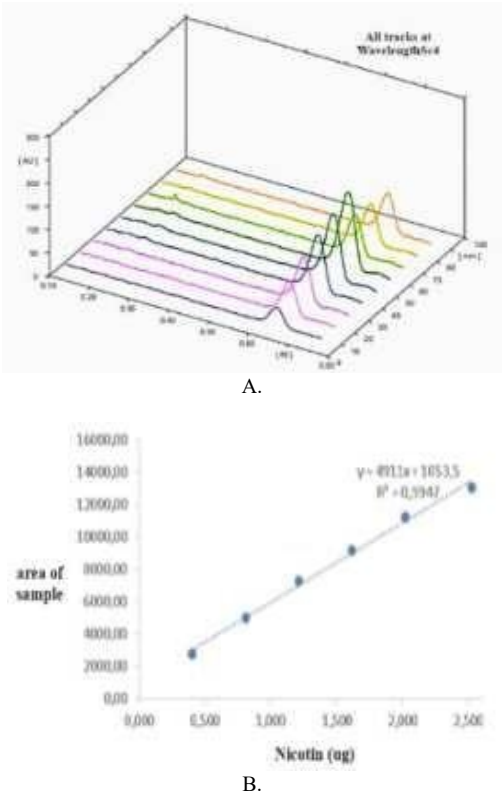



**Figure 2. Chromatogram, and Standard curve for nicotine levels.** A. 3-D chromatogram obtained using winCATS Planar Chromatography Manager. B = Standard curve for nicotine levels.

Nicotine levels in kretek cigarettes are presented in Table 1.

**Content of substances in kretek:**

Based on the GC-MS chromatogram, there are 99 substances measured in kretek (all types of substances are not shown in this paper). Substances contained in kretek with high amounts including ethyl-diisopropylacetamide (C10H21NO), Pyridine, 2-(1-methyl-2-pyrrolidinyl)- (C10H14N2), 3-Allyl-6-methoxyphenol (C10H12O2), Caryophyllene (C15H24), 10-Octadecenoic acid, methyl ester (C19H36O2), 1-Heptatriacotanol (C37H76O).

**Brain morphology and histological structure of the cerebellum of Sprague Dawley rats:**

Brain morphology and histological structure of the cerebellum of rats is presented in Figure 3.

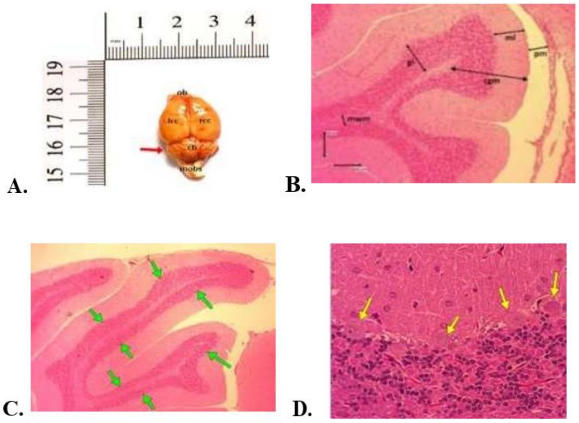



**Figure 3. Brain morphology and histological structure of the cerebellum of rats.**

A, Sprague Dawley rats9 brain. Cerebellum preparations were made in cross sections indicated by red arrows. B. Histology of the cerebel- lum of Sprague Dawley rats, hematoxylin and eosin staining, objective 10 x. ob = olfactory bulb, rcc = right cerebral cortex, lcc = left cerebral cortex, cb = cerebellum, mobs = medulla oblongata of brain stem, pm = pia matter, ml = molecular layer, cgm = cortex of grey matter, gl = granular cell layer, mwm = medulla of white matter. C. Purkinje cells at the edge of the granular cell layer (green

yellow arrow), hematoxylin and eosin staining, objective 10 x. D. Cell Purkinje (yellow arrow), hematoxylin and eosin staining, objective 40 x.

The brain morphology of Ratslooks at the olfactory bulb (ob), left and right cerebral cortex (lcc and rcc), cerebellum (cb), and medulla oblongata of brain stem (mobs). The histological structure of the cerebellum appears in the pia matter (pm), molecular layer (ml), cortex of grey matter (cgm), granular cell layer (gl), medullaof white matter (mwm), Purkinje cells at the edge of the granular cell layer (green yellow arrow). The morphology of the rat's brain between CG was no different compared to TG. Morphologically, the part of the rat's brainbetween CG was no different compared to TG. All rat brains in this study had the same parts, including ob, lcc, rcc, cb, and mobs.

***Qualitative comparison of the cerebellum between treatment groups***

Purkinje cells are located at the edge of the granular cell layer of the rat cerebellum in both groups (CG and TG). The appearance of Purkinje cells in CG appears round, while in TG they appear smaller and tapered. Based on this appearance, Purkinje cells in CG appear to have a normal histological structure, while Purkinje cells in TG appear abnormal. A comparison of the appearance of Purkinje cells in rats between groups is presented in Figure 4.

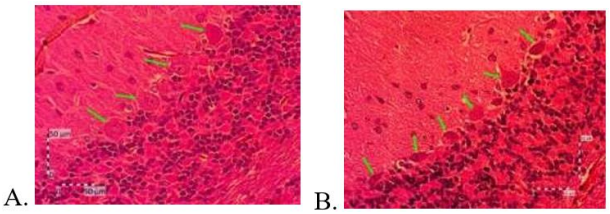
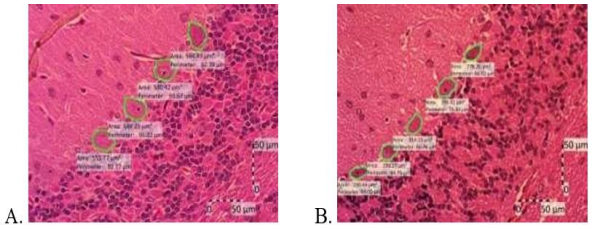


**Figure 4. Comparison of the appearance of the Purkinje cells of rats between CG compared to TG.** A. Purkinje cells in CG. B. Purkinje cells in TG. Purkinje cells in CG (A) and TG (B) stained with hematoxylin and eosin, and objective 40 x. CG, rats breathe using ordinary air without exposure to smoke of kretek. TG, the group of rats exposed to smoke of kretek 1 stick/day for 1 month (30 days) of treatment. Purkinje cells are indicated by green yellow arrows.

***Purkinje cell cytometry:***

A cytometry comparison (area, perimeter, length, and width) of the Purkinje cells of rats between CGcompared to TG are presented in Figure 5.



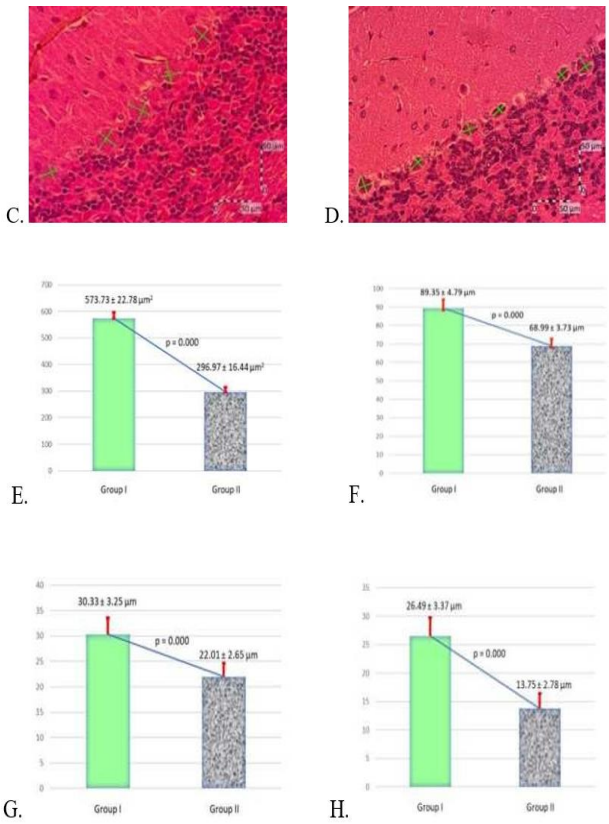



**Figure 5. Cytometry comparison of Purkinje cells in rats**

A. Area and perimeter of Purkinje cells in CG. B. Area and perimeter of Purkinje cells in TG. C. Length and width of Purkinje cells in CG (control). D. Length and width of Purkinje cells in TG. Purkinje cells in rats were stained with hematoxylin and eosin, 40x objective. E. Area comparison of Purkinje cells between CG compared to TG. F. Perimeter comparison of Purkinje cells between CG compared with TG. G. Length comparison of Purkinje cells between CG compared with TG. H. Width comparison of Purkinje cells between CG compared with TG. CG (control), rats breathe using ordinary air without exposure to kretek smoke. TG, the rat's group that exposed to kretek smoke 1 stick/day for 1 month (30 days) of treatment. Purkinje cellsare indicated by green yellow arrows.

Mean value of Purkinje cells area of rats in CG is 573.73 $\mu m^2$, with SD 22.78 $\mu m^2$, number of samples 48, with 95% lower and upper of confidence interval (CI) 567.30 and 579.93 $\mu m^2$, respectively. Mean value of Purkinje cells area of rats in TG is 296.97 $\mu m^2$; with SD 16.44 $\mu m^2$; number of samples 48, with 95% lower and upper of CI 292.46 and 301.79 $\mu m^2$, respectively. Levene's test for equality of variances of Purkinje cells area with F value 4.704; p = 0.033; p value of t-test for equality of means 0.000; with t = 68.26; df = 85.51. Purkinje cells area of rats in CG was higher than TG (p = 0.000).

Mean value of Purkinje cells perimeter of rats in CG is 89.35 µm, with SD 4.79 µm, number of samples 48, with 95% lower and upper of CI 87.98 and 90.63 µm, respectively. Mean value of Purkinje cells perimeter of rats in TG is 68.99 µm; with SD 3.73 µm; number of samples 48, with 95% lower and upper of CI 67.93 and 70.02 µm, respectively. Levene's test for equality of variances of Purkinje cells area with F value 2.70; p = 0.104; p value of t-test for equality of means 0.000, with t = 23.24; df = 94. Purkinje cells perimeter of rats in CG was higher than TG (p = 0.000).

Mean value of Purkinje cells length of rats in CG is 30.33 µm, with SD 3.25 µm, number of samples 48, with 95% lower and upper of CI 29.43 and 31.23 µm, respectively. Mean value of Purkinje cells length of rats in TG is 22.01 µm, with SD 2.65 µm, number of samples 48, 95% lower and upper of CI 21.27 and 22.79 µm, respectively. Levene's test for equality of variances of Purkinje cells area with F value 3.79; p = 0.054, p value of t-test for equality of means 0.000, with t = 13.75; df = 94. Purkinje cells length of rats in CG was higher than TG (p = 0.000).

Mean value of Purkinje cells width of rats in CG is 26.49 µm, with SD 3.37 µm, number of samples 48, with 95% lower and upper of CI 25.56 and 27.38 µm, respectively. Mean value of Purkinje cells width of rats in TG is 13.75 µm, with SD 2.78 µm, number of samples 48, with 95% lower and upper of CI 12.95 and 14.56 µm, respectively. Levene's test for equality of variances of Purkinje cells width with F value 1.033, p = 0.312, p value of t-test for equality of means 0.000, with t = 20.21, df = 94. Purkinje cells width of rats in CG was higher than TG (p = 0.000).

## DISCUSSION:

The nicotine content in kretek used in this study was 3.88 ± 0.05 µg/g. It has been shown that tobacco use is associated with nicotine dependence.[19] It has been proven that nicotine causes addiction in users.[20] The consequence of a smoker is that the nicotine levels in the body increase. Although there are other alternatives in the form of electronic cigarettes which are intended to reduce nicotine levels. [21,22,23] However, it is important to note that e-cigarettes can also use nicotine as a vaporizable substance.[24] Reducing nicotine levels in kretek needs to be done because it has the potential to provide benefits to public health.[25,26, 27] Despite the occurrence of hyperexcitability reactions,[28] smoking cessation is better for maintaining better health. Therefore, nicotinereplacement therapy (NRT) is needed to replace nicotine from cigarettes used by smokers.[29] It has been clearly stated that cigarettes cause health problems not only in the respiratory tract but also in body organs up to the molecular level, for example in antioxidant enzymes.[30] Regarding the substance content in kretek, in this study, we did not measure eugenol levels. The results of other studies demonstrate that the eugenol levels in kretek range from 2.8-33.8 mg/stick.[6]

There are several substances in kretek that are contained in high amounts, including Pyridine, Caryophyllene ($C_{15}H_{24}$), methyl ester ($C_{19}H_{36}O_2$), and 1-Heptatriacotanol ($C_{37}H_{76}O$). Apart from that, there are also molecules of ethyl-diisopropylacetamide ($C_{10}H_{21}NO$), 3-Allyl-6-methoxyphenol ($C_{10}H_{12}O_2$), 2-(1-methyl-2- pyrrolidinyl)- ($C_{10}H_{14}N_2$), and 10-Octadecenoic acid. Although we did not check eugenol levels, the kretek used in this study contained caryophyllene. We know that caryophyllene is an essential herbal oil, including clove oil. Caryophyllene has been proven to be anti-inflammatory by inhibiting key inflammatory mediators, for example inducible Interleukin 1 beta (IL-1β) and nitric oxide synthase (iNOS).[31, 32] Pyridine and its derivatives are heterocyclic compounds with low molecular weight. Even though our research results show high levels in cigarette smoke, pyridine is used as an anti-oxidant and anti-bacterial. Apart from that, pyridine is also used to treat various diseases, for example cardiovascular disease, anti-cancer, and anti-coagulant.[33] Based on the results of previous studies, we suggest that further research be conducted on the relationship between the content of substances in kretek and macrophage recruitment as an inflammatory response due to exposure to cigarette smoke.[34]

Fatty acid methyl esters (FAME) occur through the transesterification process of fat with methanol. FAME is found for example in biodiesel, usually obtained from vegetable oils through transesterification. Apart from that, FAME is also used to produce detergents and biodiesel.[35] Although it has been demonstrated that there is a correlation between fatty acid metabolic pathways and acute lung injury.[36] Because the high fatty acid content in kretek is a source of exposure to lung organs, in the future research needs to be focused on the fatty acid aspect of lung injury, as well as other organs. Heptatriacotanol molecules are also contained in kretek. Heptatriacotanol acts as an anti-oxidant, anti-inflammatory, and anti-cancer.[37]

In this study, Sprague Dawley's brain exposed to kretek smoke with low doses appeared normal, and did not differ morphologically compared to the group that was not exposed (CG as a control group). Exposure to kretek smoke with low doses, namely 1 stick/day for 1 month (30 days), did not affect Sprague Dawley's brain morphology. We compared the brain morphology of rats qualitatively. When compared with the results of previous studies, the morphology of the rat brain in this

study appears to be the same size. Moreover, it is also shown that the cerebrum appears to be the largest part of the rat brain. The olfactory bulb is in the rostral part of the cerebrum, while the caudal part of the cerebrum is the cerebellum, and the ventral part is the brainstem. [38,39]

Changes in the shape of Purkinje cells in the cerebellum of rats exposed to smoke of kretek 1 stick/day for 1 month (30 days) showed abnormalities. The Purkinje cell abnormalities in this study were like brain abnormalities in rats exposed to tobacco smoke for a long time. Previous research demonstrated that in adult rats exposed to very low doses of tobacco smoke for a long time found a reduction in immature neurons in the brain.[40] Although our previous research has not measured Purkinje cell cytometry, our study showed that exposure to smoke of cigarette 1 stick/day for 3 months decreased Purkinje cell density (in the cerebellar cortex).[16] These abnormal Purkinje cells are a sign of degeneration. Purkinje cells change size and shape, from large and round to smaller and tapered (Figure 5). The occurrence of pathological conditions of Purkinje cells in this study is in line with the oxidative stress theory. This Purkinje cell condition occurs due to oxidative stress. Oxidative stress can originate both exogenously and endogenously. Cigarette smoke contains chemical compounds that trigger compounds in cells to form proinflammatory compounds. The modulation that occurs in these intracellular proinflammatory pathways results in pathological conditions.[41] This fact is reinforced by research results that demonstrate cigarette smoke causes inflammation with various mechanisms of action.[42, 43]

The results of this research, which show a cytometry decrease in Purkinje cells in a TG are in line with the results of previous research. Previous research results that are in accordance with the results of this study include the nicotine content in tobacco. Research results have demonstrated that apart from nicotine, tobacco also contains alkaloids and other secondary metabolites.[44] In more detail, cigarettes purchased on the market contain about 1.5% alkaloids by weight of tobacco. Tobacco contributes about 95% of the total alkaloid content [45] It has been demonstrated that the nicotine in cigarette smoke decreases brain volume. It was further shown that individuals who actively smoke daily were strongly associated with a decrease in total grey matter. In more detail it was demonstrated that increasing the smoking dose had a greater effect on reducing brain volume.[46] It is not just the smoke effect of kretek on the brain, it has been further demonstrated that smoke of kretek also affects the brain reward threshold. The results of previous research on rats exposed to nicotine at various concentrations per cigarette (0.028; 0.07; 0.13; 0.272; and 0.84 mg/stick) showed that nicotine lowered the brain reward threshold.[47]

Previous research results show that nicotine in tobacco has a serious impact on the body through various multidimensional pathways. Apart from that, nicotine also has an impact on nerve health. The exact mechanism of nicotine's effect on nerve health is not yet known.[5] The influence of nicotine in cigarette smoke on neurologicalhealth is certainly related to the decreased in Purkinje cell cytometry in the results of this study. The reduction in Purkinje cell cytometry in the results of this research is in line with the results of previous studies. The results of previous research demonstrated that smoking decreased grey matter volume (GMV) in most brain areas. The GMV measurement was carried out using voxel-based morphometry (VBM). The results of this study also shown that cigarette smoke reduces GMV in the cerebellum.[48] Our results are in line with other studies showed that exposure to kretek smoke can impact brain structure and function.[49] Moreover, it has been shown that low dose cigarette smoke exposure also demonstrated of overexpression of p53 gene.[10]

Purkinje cell abnormalities in the group of mice exposed to cigarette smoke need to be compared with the control group, this is important in histopathology. Changes in Purkinje cell size include changes in area, perimeter, length, and width. The appearance of abnormal Purkinje cells is characterized by a smaller size than normal ones, so that the area, perimeter, length, and width are also reduced. Likewise, the tapered appearance of Purkinje cells also shows that the size of the area, perimeter, length, and width decreases. Quantitative changes to Purkinje cells (area, perimeter, length, and width) affect the qualitative appearance of the cells. Based on the cerebellum is a brain region that plays an important role in motor control,[12] then in the future it is necessary to investigate the relationship between Purkinje cell abnormalities and motor function.

**LIMITATION:**

The limitation of this research is that we did not measure the substance content in kretek smoke, for example levels total alkaloids, tar, and eugenol. These substances certainly affect the cerebellum of mice, especially the occurrence of Purkinje cell abnormalities. We also did not correlate the levels of substances in kretek smoke with the degree of Purkinje cell abnormalities. Apart from that, we also did not measure the thickness of each layer of the cerebellum, the characteristics of the cells in the granule cell layer of the cerebellum. Abnormalities in the size and shape of Purkinje cells which are associated with motor function are important to study because they can be used in diagnosis both at the cellular and tissue levels.[50,51]

## CONCLUSION:

Exposure to low doses of kretek smoke of 1 stick/day for 1 month (30 days) in rats did not affect morphological changes in the brain. Kretek smoke 1 stick/day for 30 days reduces the size of Purkinje cell bodies and changes the shape from round to tapered. In the future, it is necessary to investigate the clinical implications of Purkinje cell abnormalities on motor function due to kretek smoke exposure. More specifically, it is necessary to examine abnormalities in the size and shape of Purkinje cells that experience degeneration due to kretek smoke exposure.

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## CONFLICT OF INTERESTS:

All authors declare that there is no conflict of interest.

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## AUTHORS CONTRIBUTIONS:

DT, EP, HJE = Schemed, and designed experiment. DV, HJE, EP, AVO, SK = data collecting, analysis, and the results interpretation. EP, JJNT, LG, HJE = images review, and processing. DV, HJE, EP, SK = preparation of writing a manuscript. All author9s = reviewing and approved the final manuscript.

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