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Original Research Article

The Effect of Roselle Flower Infusion (*Hibiscus sabdariffa*) on Retinal Ganglion Cell Apoptosis of Sprague Dawley Rats Exposed to Cigarette Smoke

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Abstract

Background: Cigarette smoke contains a lot of free radicals that can reduce antioxidants in the body. One of those is Reactive Oxygen Species (ROS) which can induce Retinal Ganglion Cell (RGC) apoptosis. Reduced ganglion cell axons will eventually lead to optic neuropathy. Supplementing herbal medicines, vitamins, foods, and drinks containing antioxidants is one way to increase oxidant levels in the body. Roselle or *Hibiscus sabdariffa* is a herbal plant reported to have a lot of antioxidant content.

Objective: The study aimed to prove that Roselle flower infusions can reduce retinal ganglion cell apoptosis of Sprague Dawley rats exposed to cigarette smoke.

Methods: This was an experimental study with a post-test-only design with a total of 24 male Sprague Dawley rats exposed to four non-filtered cigarettes/day for 30 consecutive days. They were randomly divided into four sub-groups, and each group consisted of 6 rats. The control group received 1 ml of saline, and three treatment groups received Roselle flower infusions of 2.5%, 5%, and 10% for 30 days. Enucleated eyes were then made paraffin blocks and slides with HE staining. The retinal ganglion cell apoptosis score was assessed by calculating the number of apoptotic cells using 1000x in five fields of view. The data were analyzed using Kruskal-Wallis non-parametric test followed by the Mann-Whitney U test.

Results: The average retinal ganglion cell apoptosis score in control, treatment 1, 2, and 3 groups were 3.00; 2.83; 2.00; 1.83. There was a significant difference between the control group and treatment groups 2 (p=0.001) and group 3 (p=0.001), while between the control group and treatment group 1, there was no significant difference (p=0.317).

Conclusion: The administration of 5% and 10% Roselle flower infusions can reduce the apoptosis score of retinal ganglion cells in Sprague Dawley rats exposed to cigarette smoke. The higher the Roselle flower infusion concentration, the lower the apoptosis score of retinal ganglion cells.

Keywords: *Hibiscus sabdariffa, Retinal Ganglion Cell, Apoptosis, Cigarette Smoke*
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INTRODUCTION

Basic Health Research (Riset Kesehatan Dasar) 2018 reported that the prevalence of smokers in Indonesia continues to increase.

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In 2013, the prevalence of smokers was 29.3%, with the number of smokers in men at 47.5% and women at 1.1%. Whereas in 2018, the prevalence of smokers has increased to 33.8%, with 62.9% male and 4.8% female smokers.[1] Indonesia is the third country with the highest number of smokers worldwide after China and India.[2]

Tobacco, the main ingredient of cigarettes, contains about 4000 elements, and approximately 200 are harmful to health.[3] Toxic substances in tobacco include nicotine, tar, formaldehyde, cadmium, ammonia, cyanide acid, nitrogen oxides, pyridine, methanol, and eugenol.[4] These components can enter the body through the skin, lungs, and mucous membranes (oral and nasal mucosa).[5]

With many toxic substances in tobacco, smoking can cause several diseases, one of which is in the eyes. Tobacco consumption can cause toxic optic neuropathy. Toxic optic neuropathy is a condition, characterized by painless bilateral symmetrical visual loss, scotoma visual field defects, and color vision deficits.[6,7] This condition is typically associated with exposure to toxic substances obtained in the workplace, consumption of food or substances containing toxic substances; otherwise, this condition is a result of the use of systemic drugs, too.[8] Toxic optic neuropathy can be found equally in men and women, and affect all ages and races.[9] The incidence of pure tobacco optic neuropathy was 0.77% of the total population of nearly 300,000 patients with eye disease. This can cause health and socio-economic problems despite the small incidence. Young smokers aged 20-30 are needed in essential jobs that depend on good vision, such as in the military, civil work, and electronic industries.[10] Tobacco optic neuropathy is most often presented in elderly pipe-smoking men; however, it was also reported in cigar smokers and chewing tobacco and snuff users.[11] Color vision deficit as one of the toxic optic neuropathy conditions can occur in smokers who have smoked at least 20 cigarettes a day for eight years.[12]

The main cause of toxic optic neuropathy triggered by tobacco is a deficiency of vitamin B complex, especially thiamine (vitamin B1) and cyanocobalamin (vitamin B12). Deficiencies of riboflavin (vitamin B2), niacin (vitamin B3), pyridoxine (vitamin B6), and folic acid also play a role in this damage.[13] Chronic nicotine exposure causes vitamin B12 and folic acid deficiency, which results in the accumulation of formic acid. Formic acid inhibits electron chain transport and causes mitochondrial dysfunction; consequently, Adenosine Tri Phosphate (ATP) production decreases and the ATP-dependent axon transport system is damaged. As a result, Reactive Oxygen Species (ROS) are accumulated, which induces ganglion cell apoptosis. Reduced ganglion cell axons will eventually lead to optic neuropathy.[8,13]

Cigarette smoke contains a lot of free radicals that can reduce antioxidants in the body. Moreover, it eventually can trigger oxidative stress. The free radicals in cigarette smoke are Reactive Oxygen Species (ROS), hydrogen peroxide, and hydroxyl radicals.[14,15]

One way to suppress the levels of free radicals in the body is to increase the body's antioxidant levels.[16] Supplementating herbal medicines, vitamins, foods, and drinks that contain antioxidants is one way to increase oxidant levels in the body.[17,18] Roselle or *Hibiscus sabdariffa* is a herbal plant reported to have a lot of antioxidant content. Roselle contains vitamins, minerals, and bioactive components such as organic acids, phytosterols, and polyphenols.[19] Roselle flower petals contain antioxidants in the form of anthocyanin pigments which are a group of flavonoids.[20,21] The Roselle extract antioxidant mechanism may be due to scavenging ROS and free radicals, xanthine oxidase inhibition, lipid peroxidation reduction and elevation of antioxidant enzymes activities. Interestingly, Roselle also has antiapoptotic potential via increasing cell viability and decreasing cell apoptosis of PC12 cells under serum/glucose deprivation conditions.[22,23]

The red Roselle flowers can be dried and brewed to make herbal drinks that have antioxidant properties.[22,24] In the previous study, the toxicity dose for Roselle flower extract was 1.15 g/kg b.w.[22] In addition to having an antioxidant function, Roselle has other beneficial effects such as antibacterial, anti-inflammatory, antidiabetic, and antihypertensive.[19] There has been no study examining the effectiveness of the Roselle flower on Retinal Ganglion Cell (RGC) apoptosis. Therefore, we are interested in researching the effect of repeatedly giving Roselle flower extract as a substance rich in antioxidants on retinal ganglion cell apoptosis of Sprague Dawley rats exposed to cigarette smoke. We hypothesized that the Roselle flower could reduce retinal ganglion cell apoptosis of Sprague Dawley rats exposed to cigarette smoke.

MATERIALS AND METHODS

This experimental study with a post-test-only design was carried out at the Satmoko private laboratory, the Akurat private laboratory, and the animal laboratory in the Faculty of Medicine, Universitas Diponegoro. The research was conducted from July to September 2020. The sample of this study was the eyes of Sprague Dawley rats that had been exposed to cigarette smoke. The inclusion criteria were male Sprague Dawley rats with body weights of 200-300 grams and ages 2-3 months. The rats would be excluded as the subject if they have anatomical abnormalities, and on visual observation, the rats looked sick and/or inactive (weak). The rats would be dropped out if there was a change in behavior (weak, did not want to eat) or died during the experiment. The sample size was determined using the Federer formula, with six samples in each group. There were four groups in this study, consisting of one control group and three treatment groups that received 2.5%, 5%, and 10% Roselle flower infusions.

The infusion of Roselle was made using the infundation method. Roselle flower extract was made with a concentration of 2.5%, 5% and 10% (w/v). The preparation of 10% (w/v) Roselle infusion was carried out by carefully weighing 10 grams of dry Roselle flower powder, then adding two times the weight of aquadest and mixing it with 100 ml of aquadest. The mixture was heated for 10 minutes at 85°C with occasional stirring. The heated infusion was filtered with a flannel cloth, and hot water was added until it reached 100 ml. The making of stock infusion of 2.5% (w/v) and 5% (w/v) was done by diluting the stock infusion of 10% (w/v) using aquadest. The prepared Roselle flower infusion was given to rats with a volume of 1 ml/100gr b.w.

Table 1. Distribution of the retinal ganglion cell apoptosis score in each group

Group	Apoptosis Score				Apoptosis Score Average	p-value
	Score 0	Score 1	Score 2	Score 3		
Control	0 (0%)	0 (0%)	0 (0%)	6 (100%)	3	p < 0.001*
Treatment 1	0 (0%)	0 (0%)	1 (16.7%)	5 (83.3%)	2.83	
Treatment 2	0 (0%)	0 (0%)	6 (100%)	0 (0%)	2	
Treatment 3	0 (0%)	1 (16.7%)	5 (83.3%)	0 (0%)	1.83	

Note: Kruskal Wallis test, * Significant (p < 0,05)

Table 2. The results of the Mann-Whitney Test of the apoptosis score between groups

Group	Control	Treatment 1	Treatment 2	Treatment 3
Control	-			
Treatment 1	0.317	-		
Treatment 2	0.001*	0.005*	-	
Treatment 3	0.001*	0.006*	0.317	-

Note: Mann-Whitney Test, *significant (p<0,05)

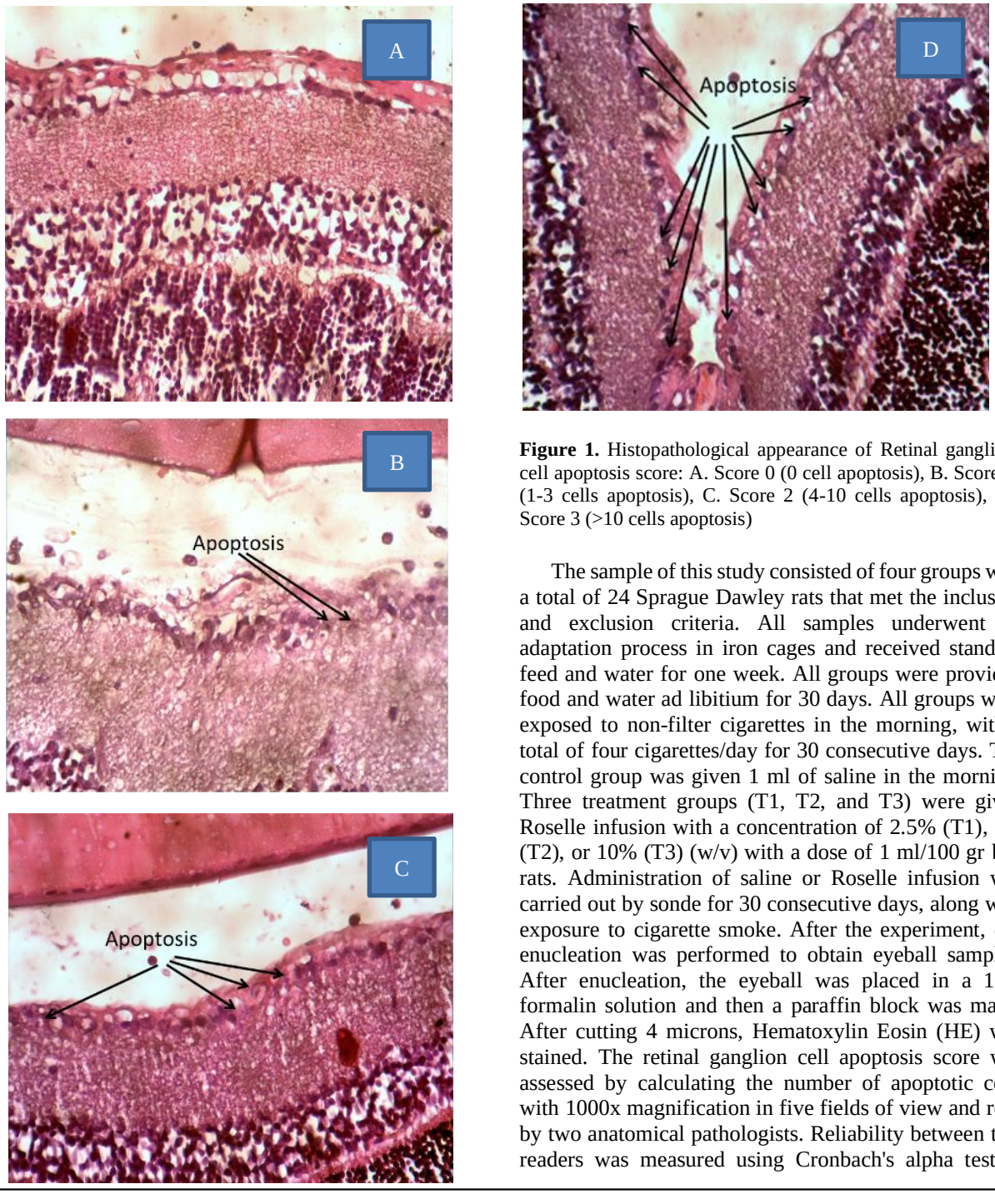

Figure 1. Histopathological appearance of Retinal ganglion cell apoptosis score: A. Score 0 (0 cell apoptosis), B. Score 1 (1-3 cells apoptosis), C. Score 2 (4-10 cells apoptosis), D. Score 3 (>10 cells apoptosis)

The sample of this study consisted of four groups with a total of 24 Sprague Dawley rats that met the inclusion and exclusion criteria. All samples underwent an adaptation process in iron cages and received standard feed and water for one week. All groups were provided food and water ad libitium for 30 days. All groups were exposed to non-filter cigarettes in the morning, with a total of four cigarettes/day for 30 consecutive days. The control group was given 1 ml of saline in the morning. Three treatment groups (T1, T2, and T3) were given Roselle infusion with a concentration of 2.5% (T1), 5% (T2), or 10% (T3) (w/v) with a dose of 1 ml/100 gr b.w rats. Administration of saline or Roselle infusion was carried out by sonde for 30 consecutive days, along with exposure to cigarette smoke. After the experiment, eye enucleation was performed to obtain eyeball samples. After enucleation, the eyeball was placed in a 10% formalin solution and then a paraffin block was made. After cutting 4 microns, Hematoxylin Eosin (HE) was stained. The retinal ganglion cell apoptosis score was assessed by calculating the number of apoptotic cells with 1000x magnification in five fields of view and read by two anatomical pathologists. Reliability between two readers was measured using Cronbach's alpha test to

obtain the kappa value. The score criteria were: score 0 = 0 cell apoptosis, score 1 = 1-3 cells apoptosis, score 2 = 4-10 cells apoptosis, score 3 = >10 cells apoptosis. The apoptosis score was calculated in each field. Determination of apoptosis score agreed by two anatomical pathologists.

The data obtained were then analyzed using the Statistic Product and Service Solution (SPSS) program version 24.0. e.g. Kruskal-Wallis non-parametric test followed by the Mann-Whitney U test. The consideration is to have statistical significance if the p-value is $p< 0.05$.

The study was conducted after obtaining ethical clearance from the Health Research Ethics Commission of the Faculty of Medicine, Universitas Diponegoro /Dr. Kariadi General Hospital Semarang for all experimental animals under number 107.3/EC/H/KPEK/FK-UNDIP/VII/2019.

RESULTS

Enucleated eyes were then put within a jar to make paraffin blocks and cut to make 24 eye slides. The reliability test (kappa value) between 2 Anatomical Pathologists by Cronbach's Alpha test was 0.99 or 99%, showing a high inter-rater agreement. The average retinal ganglion cell apoptosis score in the control group has the highest score, while the treatment group 3 has the lowest score. (Table 1).

The histopathological picture of the retinal ganglion cell apoptosis score can be seen in Figure 1.

There was a significant difference ($p<0.001$) in retinal ganglion cell apoptosis score between all groups using the Kruskal Wallis test. The difference test between groups using the Mann-Whitney test showed a significant difference between the control group and treatment groups 2 ($p=0.001$) and 3 ($p=0.001$). However, there was no significant difference between the control group and treatment group 1 ($p=0.317$).

The apoptosis score of retinal ganglion cells was significantly different between treatment group 1 and treatment group 2 ($p=0.002$) and 3 ($p=0.002$). Meanwhile, there were no significant differences between treatment groups 2 and 3 (Table 2). From Table 2, we can see that 5% and 10% concentrations of Roselle flower infusion significantly reduced retinal ganglion cell apoptosis. Meanwhile, in the control group and 2.5% concentration of Roselle flower infusion, there was no significant result in reduced retinal ganglion cell apoptosis.

DISCUSSION

The average retinal ganglion cell apoptosis score in the treatment 1 (2.5%), 2 (5%), and 3 (10%) were 2.83; 2.00; 1.83. This result shows that the higher the Roselle flower infusion concentration, the lower the apoptosis score of retinal ganglion cells. However, significant differences were only found in the treatment group that received 5% ($p=0.001$) and 10% ($p=0.001$) Roselle flower infusion. Thus, in this study, the concentration of Roselle flower infusion that can reduce ganglion cell apoptosis starts from a 5% concentration. In the control group, namely the group of rats that were only exposed to cigarette smoke, the average retinal ganglion cell apoptosis score was the highest (3.00). This result proves that exposure to cigarette smoke can cause optic neuropathy, as seen from the number of retinal ganglion cells undergoing apoptosis. This is similar to a study by Lee et al., where apoptosis of Retinal Ganglion Cells was significantly increased by exposure to Cigarette Smoke Extract (CSE); the concentrations were greater than 0.05%.[25] Grzybowski and Holder speculated that tobacco-induced optic neuropathy is a direct toxic effect on the optic nerve, especially in heavy active smokers.[5] According to a study conducted by Mochos et al., Ganglion Cell Complex (GCC) thicknesses were significantly reduced in smokers compared to the control or non-smoker group.[26] It is similar to a meta-analysis study by Yang et al. that-mention the effect of smoking on retinal and choroidal thickness. This study concluded that smoking could affect Retinal Nerve Fiber Layer (RNFL) and Ganglion Cell Layer (GCL) thickness. [27]

Management of toxic optic neuropathy due to tobacco includes cessation or reduction of tobacco consumption and/or smoking. In addition, it can be combined with a diet of green vegetables and fruits, as well as vitamin B supplementation.[13] Smoking can cause an increase in total oxidant status and oxidative stress score. Smokers also have lower total antioxidant status, vitamin E, and vitamin C compared to the non-smoker group.[28] Administration of antioxidants seems to overcome the damage caused by tobacco.

Nevertheless, this study did not examine serum antioxidant levels that can be used as the basis for the mechanism of decreasing retinal ganglion cell apoptosis given Roselle flower infusion. In this study, there were no side effects from giving Roselle flower infusion. So far, no studies have examined the effectiveness of Roselle flower infusion with toxic optic neuropathy, including those caused by tobacco.

The pathophysiology of optic neuropathy due to tobacco is currently unclear. Chronic nicotine exposure causes vitamin B12 and folic acid deficiency, resulting in formic acid accumulation. Formic acid accumulation can cause mitochondrial dysfunction so that ATP production decreases and axon transport is disrupted. As a result of all this, there is an accumulation of ROS that induces retinal ganglion cell apoptosis.[13] However, our study did not examine the levels of substances that can cause toxicity to retinal ganglion cells; such as nicotine levels, nor did we examine oxidant, and antioxidant status, which can show the mechanism of toxic optic neuropathy and the action of Roselle flower infusion in reducing retinal ganglion cell apoptosis. This is a limitation of our study.

CONCLUSION

The 5% and 10% Roselle flower infusion administration can reduce the retinal ganglion cell apoptosis score. The higher the Roselle flower infusion concentration, the lower the retinal ganglion cell apoptosis score.

This research can be used as the basis for further research on the effectiveness of herbal medicines as a treatment for toxic optic neuropathy. Further studies should consider examining nicotine levels, as well as oxidant and antioxidant status, before and after administration of Roselle flower infusion. This is aimed to show the mechanism of toxic optic neuropathy and the

action of Roselle flower infusion in reducing retinal ganglion cell apoptosis. Further studies should also consider examining the apoptosis cell quantitatively using the TUNEL assay to get more accuracy.

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