

Histological Features of Catecholaminergic Neuron in Substantia Nigra Induced by Paraquat Dichloride (*1,1-dimethyl-4,4 bipyridinium*) in Wistar Rat as A Model of Parkinson Disease

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Abstract

Paraquat dichloride has been used by farmers as a herbicide to kill the grass. On the other hand, paraquat dichloride is harmful if enters to the body, causing Parkinson's disease, since it is disrupting dopamine production in the substantia nigra pars compacta or dopamine pathways *Nigro striatal pathway*. The study was done to find out the histological changes of catecholaminergic neurons and *Nigro striatal pathway* caused by paraquat dichloride treatment in Wistar rats as a model of Parkinson's disease.

Twenty-two Wistar rats 3,5 months old were divided into 4 groups, 5 rats each. Group I (control group) were injected with aquabidest, while groups II, III, and IV were injected intraperitoneally with paraquat dichloride in aquabidest, with the dosage 5, 10 and 15 mg/kg bw respectively. The rats were injected once per week for 6 weeks. Three days after the last injection, the rats were anesthetized using xylasin (2 mg/kg) and ketamine (20 mg/kg) intramuscularly, and then were intracardiac perfused using physiological saline as preinse solution, followed by 10% buffered formalin solution as a fixative. After animals were fixed, the brains were removed and embedded in paraffin block and cut in 12 μ m thickness for immunohistochemistry staining using tyrosine hydroxylase antibody. The results of staining then were observed under light microscope and analyzed descriptively.

The results showed that the catecholaminergic neurons were distributed in the substantia nigra pars compacta in all treatment groups, however, the cell density were found decreased only in group IV. Catecholaminergic neurons appear in the bipolar and multipolar form, while dopamine '*Nigro striatal pathway*' was found exist in all treatment groups. From our study, histologically the decreased of catecholaminergic neurons is only found in rats that received paraquat dichloride in dose 15 mg/kg bw for 6 weeks.

Keywords: catecholaminergic neurons, paraquat dichloride, tyrosine hydroxylase, Parkinson's disease

Introduction

Parkinson's disease is a neurodegenerative disease that affects many elderly people due to the destruction of dopaminergic neurons in the substantia nigra (Uversky, 2004) or disruption of the

Nigro-striatal pathway that is the pathway of dopaminergic from the substantia nigra to the striatum (Betarbed *et al*, 2002). Previous studies have been conducted on the effects of paraquat dichloride in animal models is the toxic dose in rats ranged from 150-231 mg/kg, with a primary cause of death due to pulmonary damage (Gupta, 2007), the blood chemistry showed increased of creatinin, albumin and total bilirubin (Schober, 2004; Cicchetti, 2005). Tanner *et al*. (2011) reported that a Parkinson disease was associated

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with the exposure of paraquat to the body that causes oxidative stress in mitochondria. This research used low doses of paraquat dichloride, but in a long periods treatment to describe how the farmers suffering Parkinson disease in the field. The success of Parkinson disease therapy is very low until now, due to many factors. To answer why this situation is happen, the investigation on many aspects of the Parkinson disease mechanism is very urgent to do, so the prevention can be done from the beginning. Recently research showed that any environmental influences such as the use of chemicals in agriculture, heavy metal exposure, and genetic factors, such as *autosomal recessive juvenile*, potential causing Parkinson disease (Uversky, 2005).

In daily activities, farmers often use herbicides to kill weeds that interfere with the productivity of the crops. Paraquat dichloride (*1,1 dimethyl-4, 4 bipyridinum*) is a phytotoxic herbicide, usually used to kill or inhibit the growth of grass (Gupta, 2007). Worldwide use of herbicides occurred during era of agriculture modernization almost reached 48% of total pesticide used. Such increasing is correspond with the changing of monoculture farming pattern and agricultural mechanization due to the increasing of the wages of the farmers. However, the use of herbicides in long period is threatening the farmer it self, and one of the potential disease causes by herbicides is Parkinson's disease (Betarbed *et al*, 2002).

Parkinson's disease is a neurodegenerative disease caused by the destruction of dopaminergic neurons in the substantia nigra pars compacta (Emborg, 2004). Dopamine is a neurotransmitter of catecholamine group, and synthesis of catecholamine is requires tyrosine hydroxylase enzyme (TH), hence the presence of these enzymes in the organs/tissues can be used as a marker of the presence of catecholaminergic cell (Reiner, 1994). Microscopic image of Parkinson's disease is characterized by the formation of *Lewy bodies* which are eosinophilic in the dopaminergic neurons (Uversky, 2004; Schober, 2004).

The aim of the research is to determine the effect of some low dose of paraquat dichloride to the histological changes of catecholaminergic neurons in the substantia nigra of Wistar rat's. Tyrosine hydroxylase antibody was used as the marker of catecholaminergic neurons in the substantia nigra using immunohistochemistry staining techniques. From this research we expect to find the saver dose and period in using paraquat dichloride as herbicide to avoid Parkinson's disease. According to Andreollo *et al.* (2012), every one day of rat is equal to 30 days of the man's life. Six weeks of paraquat dichloride treatment to the rat in this study is approximately equal to 3,5 years of human life.

Materials And Methods

Materials

Twenty female Wistar rats (*Rattus norvegicus Albinus*), 3.5 months old were use as the animal model. Materials used to induce Parkinson's disease is paraquat dichloride (*1,1 'dimethyl-4, 4'bipyridinium*) diluted in aquadest. The animals were euthanazed using Ketamine and xylasin, while perfusion were done using physiological saline and buffered formaldehyde. Materials used for histology and staining process were ethanol, xylol, paraffin, *phosphate buffered Saline* (PBS), *blocking endogenous peroxidase*, the primary antibody *anti-TH mouse serum* (Immunostar, Cat. No. 22941), and kit Startrek Universal HRP Detection (Biocare Medical, USA, Cat. No. STUHRP700 L10) that consist of *blocking background* material, secondary antibody, avidin-biotin, and chromogen *3,3-diaminobenzidine tetrahydrochloride* (DAB). The equipment used were the infusion set, microtechnic set, light microscope with a digital camera.

Methods

Perfusion and preparation of the brain samples

Rats were divided into 4 groups, each group consisted of 5 animals. Group I was

the control group injected with aquabidest, group II were injected with 5 mg/kg paraquat dichloride, group III (10 mg/kg), and group IV (15 mg/kg). Rats were injected intraperitoneal once per week for 6 weeks.

Three days after the last injection, the rats were anaesthetized using combination xylasin (2 mg/kg) and ketamine (20 mg/kg) by intramuscularly injection. During anesthetized condition, the rats were perfused intracardially using physiological saline as a *pre-rinse*. Intracardiac perfusion were done by flowing *pre-rinse* solution to the left ventricle and let it out from right atrium. The fixative solution (10% buffered formalin) was flowed into the left ventricle following the *pre-rinse* after the *pre-rinse* solution that flow out from the right atrium was free from blood. The fixative solution was perfused until the whole body well fixed. Once the perfusion process completed, the brains were collected and fixed with 10% buffered formalin solution.

The brain was cut longitudinally in the mid line into two parts. The left side was cut in the coronal of mesencephalika flexure area with 5 mm thick, then were blocked using paraffin. The hemispheres then were stored for histological examination.

The process of paraffin blocking and tissue sectioning

Slices of brain were inserted into the *tissue cassette*, followed by dehydration process that carried out in serial solution of ethanol 70%, 80%, 95%, and absolute ethanol for 60 min each at room temperature. Purification (*clearing*) process was conducted by mixing xylol and 100% ethanol solution, followed by xylol twice, for 45 minutes each at room temperature. Paraffin infiltration was conducted three times, for 45 minutes each, at 60°C, then were embedded in paraffin, then cooled at room temperature.

Paraffin block were cutted in 12 µm thick using a *rotary microtome*, then placed on the surface of warm water (45°C) and placed on object glass that have been coated by gelatin and vertically drained. Glass

object then placed on a *slide warmer* until well adhered.

Immunohistochemical staining of tyrosine hydroxylase

Slides of the tissue were arranged in racks and incubated at 60°C for 2 hours, then were cooled at room temperature. Tissue was deparaffined in xylol, three times 5 minutes each and continued by rehydration process in absolute alcohol, twice in 90% alcohol, followed once in alcohol 80%, alcohol 70%, 5 minutes each. After that, the slides were dipped in distilled water for 10 dips, then washed three times in *phosphate buffered saline* (PBS), each for 5 minutes.

Endogenous peroxidase in the tissue was blocked by immersing slides in to 3% H₂O₂ for 30 minutes, then were rinsed three times in PBS, 10 minutes each. Surfaces around the slides were dried using tissue paper and keep the tissues moist. Slides then were aligned horizontally in a humid box, then tissue were spilled with *Revealit Antigen* solution and incubated at 37°C one night. Slides then were rinsed four times in PBS solution, 10 minutes each. Blocking background were done using a *sniper* for 15 min at room temperature.

Primary antibody *Rat anti- tyrosine hydroxylase mouse serum* (1/2500) were applied to the adrenal tissue of the treatment groups, while the adrenal tissue of the control groups containing tyrosine hydroxylase were not treated with primary antibody. Slides then were placed horizontally in a humid box and incubate for 2 nights at 4°C, then rinsed four times with PBS, for 15 minutes each. Furthermore, tissue was incubated at room temperature with a secondary antibody (Trek Universal Link) for 20 minutes, then rinsed three times using PBS, for 5 minutes each. Tissues were spilled with *TrekAvidin-HRP* for 10 minutes and then were rinsed three times with PBS, 5 minutes each, then incubated with *Betazoid DAB + substrate* solution for 3 minutes. Tissues then were washed in distilled water for 10 dips and

stained with hematoxylin for 1 minute as a counterstained. Finally, the tissues were dehydrated in alcohol, cleared in xylol, and coverslipped using Canada balsam.

Staining results

The results of immunohistochemical staining of tyrosine hydroxylase based on the presence of catecholaminergic neurons *Nigro-striatal pathway* then were observed under microscope, and photographed by digital camera. Immunoreacted neurons were characterized by brownish yellow color of the cell. Three slides from each brain were observed for histology examination.

Analysis of research results

The images were processed with *Adobe Photoshop 7.0 software* and analyzed descriptively.

Results and Discussion

Catecholaminergic neurons in the substantia nigra

Based on the observation, we found that catecholaminergic neurons are distributed in the substantia nigra pars compacta (SNc) both of the treated rats group and the control group. Position of SNc is in the dorsal of substantia nigra pars reticulata (SNr). Catecholaminergic neurons are immunoreacted neurons

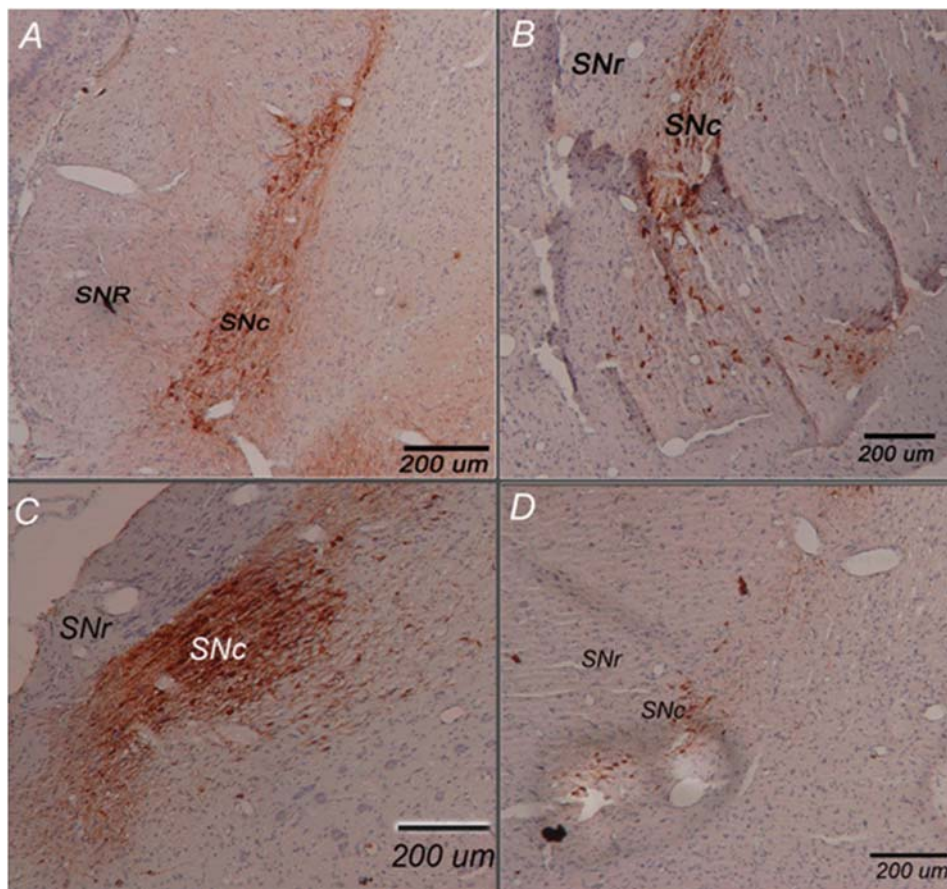


Figure 1. Catecholaminergic neurons in the substantia nigra pars compacta of rats after injected intraperitoneally with aquades, once a week for 6 weeks by tyrosine hydroxylase immunohistochemical staining. A. control's; B. dose 5 mg/kg bw's; C. dose 10 mg/kg bw's; D. dose 15 mg/kg bw's groups. Tyrosine hydroxylase immunoreacted neurons are catecholaminergic neurons, showed brown in color. SNc: substantia nigra pars compacta. SNr: substantia nigra pars reticulata.

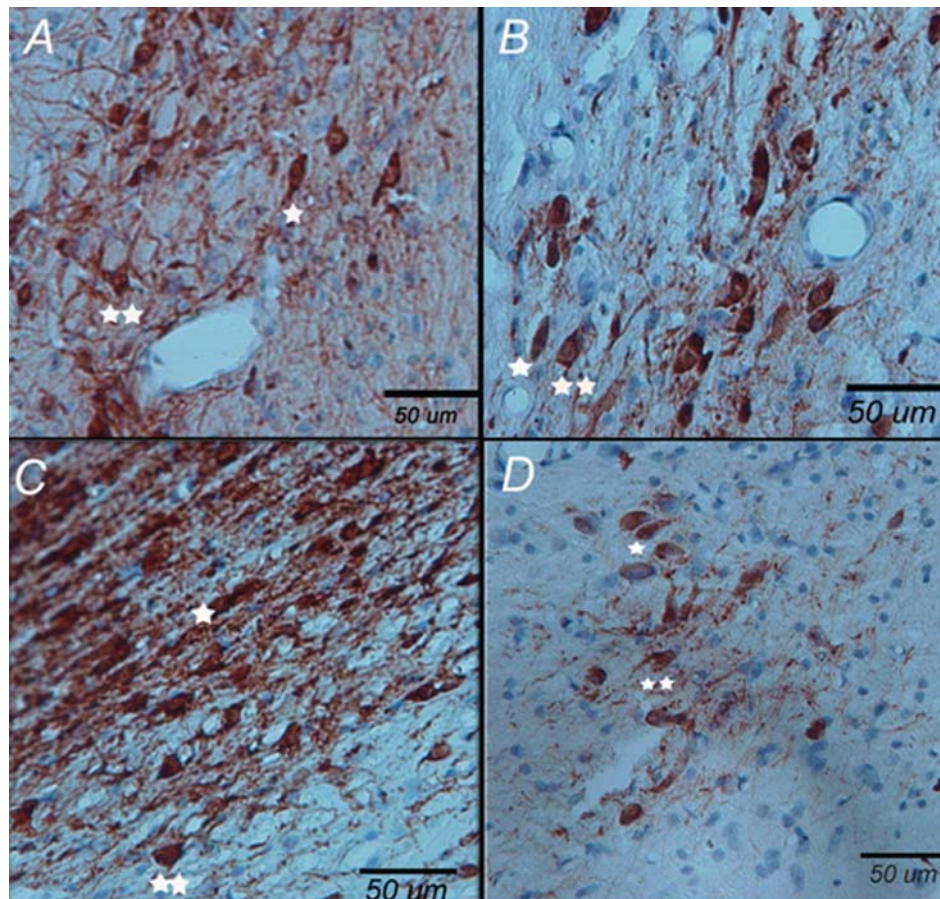


Figure 2. Type of catecholaminergic neurons in the substantia nigra pars compacta of rats after injected intraperitoneally with aquades, once a week for 6 weeks by tyrosine hydroxylase immunohistochemical staining. A. control's; B. dose 5 mg/kg bw's; C. dose 10 mg/kg bw's; D. dose 15 mg/kg bw's groups. Tyrosine hydroxylase immunoreacted neurons are catecholaminergic neurons, showed brown in color. Catecholaminergic neurons are bipolar (*) and multipolar (**) in shape. Figure D showed the decreased of neuronal density of catecholaminergic neurons compared with Figure A, B and C.

to tyrosine hydroxylase (TH) antibodies (TH) by immunohistochemical staining (Figure 1). Catecholaminergic neurons substantia nigra pars compacta are bipolar and multipolar in shape (Figure 2). The shape of Catecholaminergic neurons substantia nigra pars compacta in this research is similar with the shape of catecholaminergic neurons substantia nigra pars compacta of rhesus monkey that was reported by Felten and Sladek (1983).

Catecholaminergic neurons in rats of treatment group that injected paraquat dichloride in dose 15 mg/kg bw were showed decrease in the cell densities compare with

other groups, as shown in Figure 2D. The decrease in the cell densities are possibility the process of the cell death, as described by Bertabet (2002) is paraquat dichloride penetrate the blood-brain barrier, then metabolized into MPP⁺ metabolites that toxic to mitochondria of dopaminergic neurons in the substantia nigra pars compacta and causing disruption of respiration cells and cellular oxidative stressed. According to Miller (2007) Paraquat can inhibit complex I of cell respiration process in mitochondria.

From the case-control study by Tanner *et al.* (2011) showed that Parkinson diseases are associated with paraquat. Their suggestion

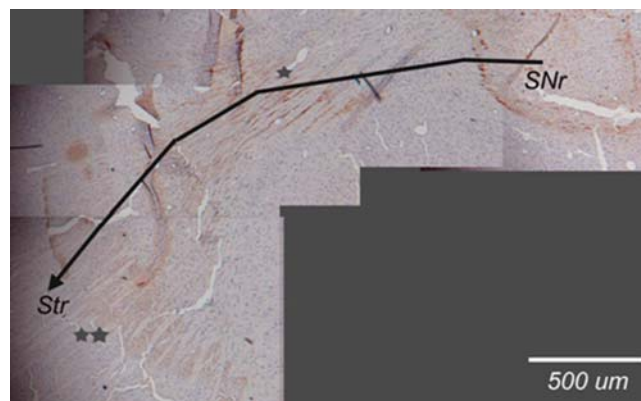


Figure 3. *Nigro-striatal pathway* expand from the substantia nigra pars compacta (SNc) to the striatum (Str) in the rats who injected with 5 mg/kg BW paraquat dichloride intraperitoneally, once a week for 6 weeks, arrows indicating Catecholamines is carried by axons (*) to the axon terminal (**).

is paraquat increases production of certain oxygen derivate that may harm cell structure. In this research it is likely, some cells unable to overcome with oxidative stress that caused cell death. Further research needs to investigate the occurrence of oxidative stress by using its specific marker.

Based on the clinical symptoms, treated rats did not showed the typical signs of Parkinson's disease such as tremor, rigidity, and difficulty moving. It is likely, the cell damaged that occurred is still able to be overcome by the body.

The existence of Nigro-striatal pathway

The dopamine pathway is expand from the substantia nigra pars compacta heading into the striatum, called *Nigro-striatal pathway* as found on the treated groups. This pathway begins with catecholaminergic neuron cell bodies that produce dopamine in the substantia nigra pars compacta, then carried by the axon toward the axon terminals in the striatum, as shown in Figure 3. Rats that received paraquat dichloride in dose 15 mg/kg bw showed the axon and axon terminal that immunoreactive to tyrosine hydroxylase antibody (Figure 4) which is indicated that *Nigro-striatal pathway* exist in this group. The existence of *Nigro-striatal pathway* showed that the dopamine system has been working properly so that the animals did not suffered

of rigidity although the group of rats that received paraquat dichloride in dose 15 mg/kg showed weaker body condition compare with other groups.

Parkinson's disease characterized by disruption of dopamine production in substantia nigra pars compacta or alteration of *Nigro-striatal pathway* and showed the clinical sign such as as tremor, bradykinesia, hypokinesia, balance and movement disorders (Paxinos, 2004; Betarbed *et al*, 2002; Emborg, 2004).

The results of this study showed that although the rats did not showed symptoms of Parkinson's disease and *Nigro-striatal pathway* is remain exist, but there is a decreased of neuronal density of catecholaminergic neurons in the substantia nigra pars compacta in the rats that reveived paraquat dichloride in dose 15 mg/kg bw. Based on this results, it is hypothetized that dose 15 mg/kg bw of paraquat dichloride treatment period caused neuronal changes of catecholaminergic neurons in the substantia nigra pars compacta and its potentially leading to Parkinson's disease.

Conclusions

From our study, histologically the decreased neuronal density of catecholaminergic neurons in substantia nigra pars compacta is only found in rats that

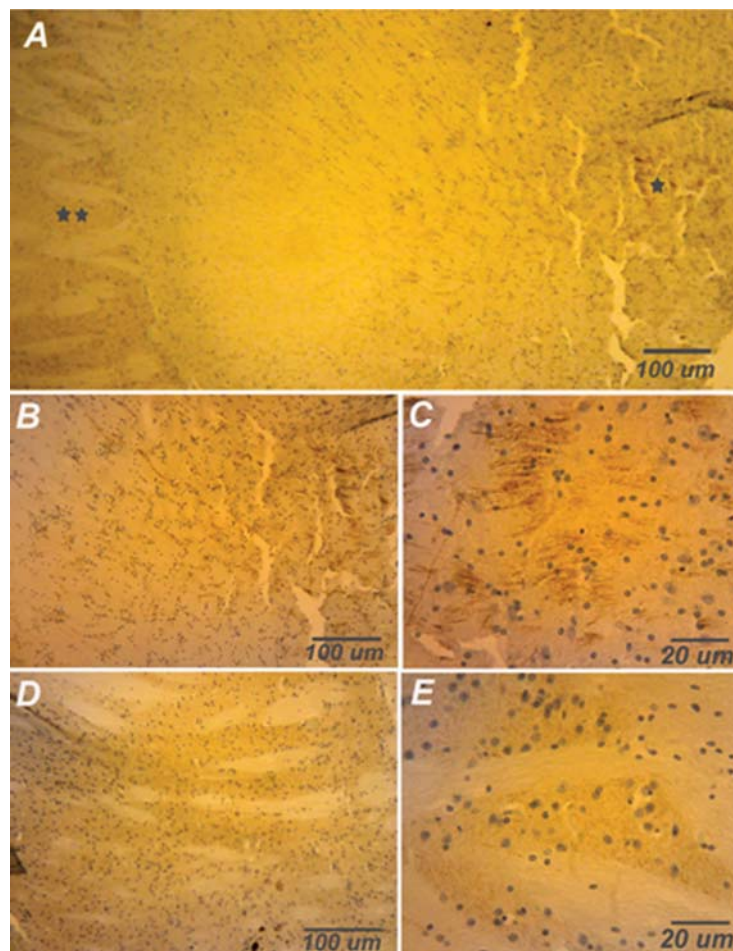


Figure 4. Axon and axon terminal of tyrosine hydroxylase immunoreactive tissue in the striatum of the rats who injected with 5 mg/kg BW paraquat dichloride intraperitoneally, once a week for 6 weeks. A. One star indicating catecholamines in the axons and two stars indicating catecholamines in the axon terminal of striatum. B and C are higher magnification of axon (one star area) that immunoreactive to tyrosine hydroxylase. D and E are higher magnification of axon terminal in the striatum (two stars area) that immunoreactive to tyrosine hydroxylase.

received paraquat dichloride in dose 15 mg/kg bw for 6 weeks.

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