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Assessing the Neurotoxicological Effect of the Acute Paraquat Aerosols Exposure in Causing Parkinsonism on Mouse through Behavioral Assays

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Abstract

***Background**: In the scientific community, there is no consensus that paraquat, a widely used herbicide, has a strong relationship with the occurrence of Parkinson's disease. A reliable epidemiological explanation of how paraquat can induce parkinsonism is urgently needed because it relates to the agriculture community's potential public health problem. **Objective**: In this study, mice exposed to aerosols of paraquat solution were assessed by behavioral assays designed to observe whether mice exposed to paraquat aerosols develop cardinal symptoms of Parkinson's disease, such as tremor-at-rest, bradykinesia, rigidity, and postural instability. **Methods**: To obtain the intended information, we carried out the observation on distal extremities, catalepsy test, wire suspension test, and swimming test consisting of the head position sub-test, the involvement of limbs sub-test, and the swimming direction test, respectively, to both the group of mice exposed to paraquat aerosols and the one which is not. **Results**: According to the result of the independent-samples t-test calculation on the data obtained from behavioral assays, a significant difference is shown only by the wire suspension test used to assess the development of forelimb rigidity and not the others. **Conclusion**: Therefore, this study showed that daily exposure for a week to paraquat aerosols insignificantly causes tremor-at-rest, bradykinesia, and postural instability in studied mice but dramatically affects their forelimb performance in the form of rigidity.*

***Keywords**: paraquat, Parkinson's disease, parkinsonism, aerosol, behavioral assay*

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INTRODUCTION

To eradicate undesired weeds and grasses from the plantation field during the planting period, farmers usually apply herbicides by spraying them in the field for a period (Konthonbut *et al*., 2020). However, in most developing countries where deficient working conditions, such as the type of equipment being used are poor, improper maintenance of equipment by farmers, climatic conditions in which farmers usually stand upwind while spraying, and illiteracy make controlled and safe use of herbicide is complex (Watts, 2011). Among the widely used herbicides in agriculture is paraquat (1,1-dimethyl-4,4-bipyridinium dichloride). As one of the highly toxic nonselective contact ammonium herbicides, paraquat is associated with the occurrence of Parkinson’s disease (Tanner *et al*., 2011: Gao *et al*., 2020), a neurological movement disorder characterized by the loss of the nigrostriatal dopaminergic pathway (Tieu, 2011).

Even though there are several pathways that paraquat can exist in the body and give rise to health problems, those are ingestion of the residue in water or food, dermal penetration, and inhalation of the aerosols, a recent finding by Anderson *et al.* (2021) showed that paraquat could be found and translocated to various brain regions of mice exposed to paraquat aerosols. It is known that paraquat can trigger oxidative stress that leads to neuronal damage (Guo *et al*., 2018). However, it is still unclear how paraquat can specifically attack dopaminergic neurons in substantia nigra pars compacta (Tieu, 2011) to induce parkinsonism.

In our laboratory, we exposed aerosols of paraquat solution onto the face of mice and isolated them for 10 minutes in an isolation cage. After daily paraquat exposure for a week, we carried out behavioral assays on studied mice to know whether acute paraquat aerosols exposure can induce the manifestation of movement disorders associated with Parkinson’s disease (parkinsonism), such as tremor-at-rest, bradykinesia (slowness of a performed movement), rigidity, and postural instability (inability to lance due to loss of postural reflexes) (Berardelli *et al*., 2001; Jankovic, 2008). In contrast to many studies on the effect of paraquat on the occurrence of Parkinson's disease, which were conducted via intraperitoneal injection, we attempted to demonstrate whether the result would be consistent if one of the actual conditions in which most farmers are exposed to paraquat in their plantation field, namely exposure to paraquat aerosols inhaled when applying that herbicide by spraying, was mimicked.

MATERIALS AND METHODS

Animals

This study is based only on BALB/c male mice 8-12 weeks in age bred at Andalas University. Mice were caged individually and provided standard rodent chow and water *ad libitum*. After being acclimatized (12 hours day and 12 hours night) for a week at Animal Anatomy and Physiology Laboratory, University of Bengkulu, mice were randomly divided into two groups containing eight mice each. The first group was the control group (P0), which was the group that is only being maintained in their cage during the study, whereas the other group was the mice exposed to aerosols of paraquat solution (P1). All mice used in this study were treated ethically with procedures approved by the Committee of Ethics at the Faculty of Medicine and Health Sciences, University of Bengkulu.

Paraquat exposure

The source of paraquat used in this study is Gramoxone containing 276 g/L paraquat dichloride (equal to 200 g/L paraquat ions) produced by Syngenta Indonesia. Before being used in the study, 5 mL of Gramoxone was mixed with aqua dest until the volume of the solution reached 500 mL. The paraquat solution was then poured into a spray bottle.

Paraquat exposure was conducted by spraying the aerosols of paraquat solution onto the face of each P1 mouse three times (±2.62 mL of paraquat solution received by each mouse) in the isolation cage. After 10 minutes of isolation, each mouse will be placed back into its maintenance cage. This procedure was done daily for a week for P1 mice.

Behavioral assays

Behavioral assays were done in a quiet, dimly lit room in the evening time three days after the last exposure of paraquat aerosols applied to P1 mice. Behavioral assays that were carried out in this study consist of the catalepsy test, inclined plane test, wire suspension test, and swimming test, which comprises a head position sub-test, limb movement sub-test, and swim direction sub-test. The detail of how each behavioral assay was done is as follows:

The catalepsy test was carried out based on Grabow & Dougherty (2001), with the scoring method used in this study as follows: 0 if the mouse went ahead toward the gap when it was placed at the edge of the table; 1 if the mouse kept staying in its position at the edge of the table more than 10 seconds; and 2. if the mouse turned backward in less than 10 seconds to avoid the gap.

The wire suspension test was performed by allowing the mouse grasps a horizontal wire and then evaluating its ability to pull its hanged body. It got 0 if it could not hold the wire provided to it and pull its hanged body up by flexing its forelimbs, and one if vas versa.

The inclined plane test was conducted based on Grabow & Dougherty (2001), with the scoring method as follows: 0 if the mouse slipped down from the inclined plane; 1 if the mouse could keep its body in the inclined plane; and 2, if the mouse was not only able to keep its body in the inclined plane but also performed negative geotaxis movement.

The swimming test was conducted by placing the mouse in a water-filled aquarium and evaluating the response shown by the mouse while in the water. This test observed three aspects: head position, swimming direction, and whether the mouse uses its limbs to swim. For evaluating *the head position*, the mouse would get 0 as its score if the entire head of the mouse sank in the water; 1 if the nose of the mouse was above the surface of the water; 2 if the nose and upper head of the mouse were above the surface of the water; 3 if nose and eyes, as well as ¾ of mouse's ears, were above the surface of the water; and 4, if nose, eyes and entire ears of the mouse were above the surface of the water. For evaluating *the swimming direction*, the mouse would get 0 as its score when the mouse sank, 1 when the mouse floated in the water, 2 when the mouse swam circularly, and 3 when the mouse straightly swam toward its desired direction. For evaluating *the involvement of limbs*, the mouse would get 0 if the limbs were not involved in swimming; 1 if only hindlimbs were involved; and 2 if both fore- and hindlimbs were involved.

Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics version 28.0.1.1 (IBM SPSS, Inc,) for Windows. Statistically significant differences between two normally distributed groups were analyzed by performing the Independent-Samples T-Test, in which statistical significance was set at $p < 0.05$. All data were presented as mean + standard deviation.

RESULTS

A slight, insignificant decline in the mean score of the catalepsy test for the group of mice exposed to paraquat

The catalepsy test is a behavioral assay designed to assess whether mice lose spontaneous mobility (Simon *et al*., 1970). The loss of spontaneous movement indicates that the mice have difficulty initiating a movement and/or slowly performing it (Jankovic, 2008). Therefore, this test could show whether mice exposed to paraquat aerosols in this research developed bradykinesia.

As illustrated by Figure 1a, paraquat aerosol exposure could slightly decrease the score for the catalepsy test of the mice exposed to paraquat compared to the control group. However, the result of the independent-sample t-test ($p = 0.149$) shows that there is no significant difference between the mean score of paraquat-exposed mice and the control group ($p > 0.05$).

A significant decrease in the mean score of the wire suspension test for paraquat-exposed mice

Rigidity in Parkinson's disease manifests in flexor muscle inhibition and extensor muscle facilitation (Andrews *et al*., 1972). In this study, the wire suspension test score represents the ability of a mouse to flex its forelimbs when it pulls up its body which hung with its forelimbs grasp a string of wire. Figure 1b shows that the mean score of the wire suspension test in paraquat-exposed mice highly decreases compared to the control group's mean score. Further independent-sample t-test on this data ($p = 0.004$) confirmed that the difference between the mean score of both the control and paraquat-exposed group is significant ($p < 0.05$).

A statistically insignificant decline was observed in the mean score of the inclined plane test for mice exposed to paraquat

To maintain the balance of the body in an inclined plane, mice need to have good postural reflexes, and to assess whether paraquat-exposed mice lose their postural reflexes, this research observed the response of both the group of mice exposed to paraquat aerosols and the control group when they were placed in an inclined plane. Figure 1c presents the bar graph of the mean score of both groups, showing that paraquat exposure could decline the score of the inclined plane test result in several mice exposed to paraquat aerosols, even though if it was compared to the control group, the difference between both groups is statistically insignificant ($p = 0.060$) based on the independent-samples t test calculation ($p > 0.05$).

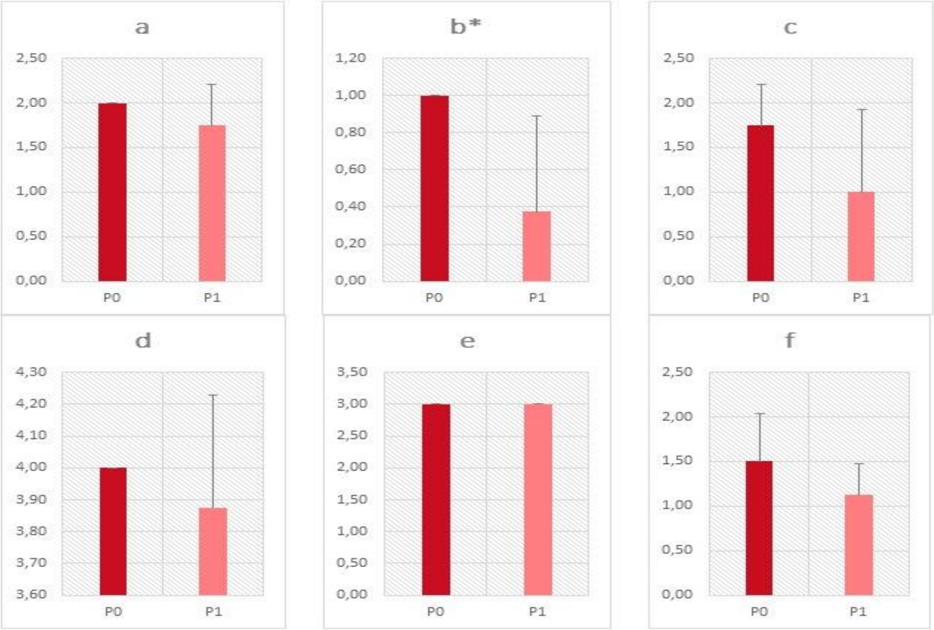

Figure 1. The result of behavioral assays of mice exposed to paraquat aerosols (P1, pink) and the control group (P0, red). Each bar represents the mean score of the assessed behavioral assays (see Materials and Methods). Behavioral assays tested for both groups comprises the catalepsy test (a), wire suspension test (b), inclined plane test (c), and swimming test. The swimming test assessed for both groups consists of the head position sub-test (d), swimming direction sub-test (e), and limbs-using sub-test (f). Error bars on the graphs show the standard deviation. Asterix sign means the test result shows a significant difference statistically

An insignificant difference in the result of the swimming test between the group of mice exposed to paraquat aerosols and the control group

Swimming is a complex movement that includes the ability to coordinate each limb to keep the body's balance and move the body to the desired direction while in water. In this study, the ability to maintain body stability in water was assessed by scoring the ability of a mouse to keep its head out of the water, whereas the ability to perform a movement at a required speed to avoid drowning was assessed by scoring the ability to swim toward a certain direction. Furthermore, to check whether the mouse can easily use its limbs for swimming properly, we observe the movement of limbs during swimming activity.

Each behavioral assay score represents the mouse's ability to conduct normal movements required to swim when the mouse does not develop parkinsonism. When a mouse swims and can maintain its head out of the water, it is more likely to have no problem balancing its posture in the water and vice versa. Moreover, if the mouse has no problem swimming properly toward any direction it wants, it means the mouse does not develop bradykinesia because if it does, the mouse will be drowning. The ability of the mouse to move its limbs when it swims will inform that its flexor and extensor limb muscles can function properly, indicating that there is no rigidity.

As depicted by Figure 1d, the mean score for the observation of mouse head position is slightly decreased in mice exposed to paraquat aerosols compared to the control group, which was confirmed insignificant ($p > 0.05$) by the independent-samples t-test calculation ($p = 0{,}334$). In the ability to swim properly in any direction, both the control group and mice exposed to paraquat show the exact same mean score (Figure 1e). For the result of the use of limbs sub-test (Figure 1f), the statistical calculation ($p = 0.120$) proved that the difference between the control group and the group of mice exposed to paraquat aerosols is insignificant ($p > 0.05$).

There is no observed tremor-at-rest in the group of mice exposed to paraquat aerosols

Tremor-at-rest, or rest tremor, is the most common and easily recognized symptom of Parkinson’s disease and almost always be prominent in the distal part of extremities. Observation on the distal part of mice's extremities was done during this research, and there is no observed tremor-at-rest in both mice exposed to paraquat aerosols or the control group.

DISCUSSION

Many studies showed that cardinal symptoms of Parkinson's disease only emerge when the dysfunction of the basal ganglia circuit involved in motor control, including locomotor movement and posture, ensued from the degeneration of 50-80% of substantia nigra dopaminergic neurons, which are part of the input level of the circuit (Grillner *et al*., 2013; DeMaagd & Philip, 2015; Yttri and Dudman, 2018). Tremor-at-rest, for example, is a condition caused by the failure of the circuit's direct pathway output level (which includes the GABAergic substantia nigra pars reticulata and the globus pallidus internal) as a result of the dysfunctional input level, with the effect on the indirect pathway output level (which includes the subthalamic nucleus) causing bradykinesia (Wichmann & DeLong, 1996; Tai *et al.*, 2012; Grillner *et al*., 2013; Yttri & Dudman, 2018). Moreover, because of the reciprocal connection between the basal ganglia and the mesencephalic locomotor region cholinergic neurons involved in the control of locomotion, posture, and balance, the dysfunction of the basal ganglia circuit also gives rise to postural instability in Parkinson's disease patients (Grabli *et al.*, 2012; Pahapill & Lozano, 2000; Caggiano *at al*., 2018).

In the previous study by Fahim *et al.* (2013), rats which were administered by intraperitoneal injection of paraquat for three weeks in a row showed a significant reduction in motor activity and difficulty in movement following the degeneration of dopaminergic neurons in substantia nigra pars compacta. However, our study on mice exposed to paraquat aerosols for a week showed a different result. As shown in Figure 1 (except Figure 1b), the mean score for behavioral assays designed to assess mouse motor performance in this study showed a statistically insignificant reduction or none in the group of mice acutely exposed to paraquat aerosols compared to the control group. This result may imply that the typical degeneration of dopaminergic neurons in substantia nigra that led to a significant reduction in motor activity and difficulty in movement is absent in mice exposed to paraquat aerosols in our study. Furthermore, it also suggests that the degree of paraquat neurotoxicity may depend on the route paraquat comes into the body, how a body of different species deals with it, and the duration of exposure, since those three aspects are what differs our study from Fahim *et al.* (2013).

However, the result of the wire suspension test for both the control group and the group of mice acutely exposed to paraquat aerosols in this study (Figure 1b) is prominent evidence that paraquat can induce neuronal damage because it can cause rigidity to emerge in the forelimb of paraquat aerosols exposed mice. One of the possibilities underlying rigidity in Parkinson's disease is brainstem degeneration (Bologna & Paparella, 2020).

A study by Esposito *et al.* (2014) found that the brainstem nucleus medullary reticular formation ventral part, which its high synaptic density was found contacts to the biceps, extensor carpi radialis, and extensor digiti quarti motor neurons, is a key brainstem area specifically connecting to a subset of forelimb-innervating spinal motor neuron. Degeneration of those brainstem nuclei might affect forelimb motor performance, such as causing rigidity. Therefore, the result of our study in which forelimb rigidity is observed also supports the current view that was established by the study by Braak *et al.* (2003), that the early stage of Parkinson's disease is started in the brainstem and will only show its full clinical manifestation when neurodegeneration has reached substantia nigra.

However, as far as our knowledge, there is no research focused on the effect of paraquat on brainstem degeneration either anatomically or immunohistochemically, so we suggest that future research may focus on this area to fully understand how paraquat can gradually induce neuronal damage in the brain leading to Parkinson's disease. Moreover, since farmers who worked in plantation fields are regularly exposed to herbicide throughout their lifetime, to fully understand the effect of paraquat aerosols in inducing the occurrence of Parkinson's disease, unlike this study which focused only on the impact of acute exposure, we need to know whether the chronic exposure of paraquat aerosols may, too, lead to parkinsonism.

CONCLUSION

In summary, our study shows that daily exposure for a week to paraquat aerosols insignificantly causes tremor-at-rest, bradykinesia, and postural instability in studied mice but dramatically affects their forelimb performance in the form of rigidity.

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AUTHOR CONTRIBUTIONS

Conceptualization, S. A. M.; Methodology, C. M.; Validation, S. N. K., R. R. S. A.; Formal Analysis, S. A. M.; Investigation, S. A. M.; Data Curation, S. A. M.; Writing - Original Draft, S. A. M.; Writing - Review &

Editing, S. N. K.; Visualization, S. A. M.; Supervision, C. M., A. R.; Project Administration, C. M., S. N. K.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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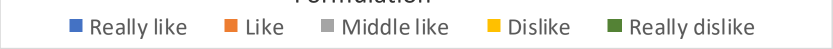

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