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ORIGINAL ARTICLE

Ataxia and Cerebellar Dysfunction in Low Dose Methylmercury-induced Balb/c Mice

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ABSTRACT

Introduction: Methylmercury (MeHg) can penetrate the blood-brain barrier and cause brain cell damage, also give impacts such as decreased vision, hearing loss, paresthesia, and ataxia. However, the impact of a low dose of MeHg exposure on neurological disorders is not well understood. This research is aimed to investigate the effect of a low dose of methylmercury (MeHg) exposure in the early symptoms and onset of neurological disorders measured by ataxia and function of cerebellum in Balb/c mice. **Methods:** This research was an experimental research with post-test only control group design. Fifteen Balb/c mice were randomly divided into 3 groups of control, MeHg1 (5 mg/kg body weight (BW)/day), and MeHg2 (10 mg/kg BW/day) all given orally treatment once a day for 17 days. Ataxia was observed using the Hind-Limb Clasping test and Ledge test methods. The cerebellum was separated and undergone Hematoxylin Eosin (HE) staining for histopathological analysis. Statistical tests used Fisher-Exact, One-way ANOVA, and Kruskal-Wallis tests. **Results:** The results showed a significant difference between the level of MeHg with the level of ataxia ($p < 0.001$; Fisher Exact test) and the number of intact Purkinje cells as an indicator of cerebellar histopathology changes ($p = 0.001$; One-way ANOVA test). The ataxia level was significantly correlated with histopathological features of the cerebellum in mice ($p < 0.05$; Kruskal-Wallis test). **Conclusion:** Low dose exposure of MeHg could induce ataxia and cerebellar dysfunction.

Keywords: Methylmercury, Ataxia, Purkinje cell, Cerebellar dysfunction

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INTRODUCTION

The exposure of mercury (Hg) in humans has become a special issue in the world because of the various effects of its exposure to human health (1). The exposure is commonly from Hg-contaminated food and industrial process such as coal-burning vapor and mining (2, 3). Indonesia has known as a developing country with many traditional gold and silver mining industries that still use Hg in their amalgamation process and included as one of a country that facing Hg pollution problem United Nations Environment Program (UNEP) (2, 3).

Mercury has 3 forms, those are elemental Hg (Hg0), inorganic Hg (Hg2+), and organic Hg or known as methylmercury (MeHg) (1). MeHg became an important neurotoxicant because can penetrate the blood-brain barrier (BBB) and cause brain cell damage (4). MeHg can be accumulated into food particularly fish and other water species and contaminated humans through the food chain (6, 7). After entering the body, MeHg will be accumulated in the kidney, liver, skin, brain, and hair (8). Therefore, the community living near the coastal area, as well as coal and gold mining areas have a high risk of MeHg exposure.

The major effect of MeHg toxicity have been reported in some exposed area such as Minamata at 1950, Nigata at 1964 Japan and Iraq at 1970, with prominent intoxication symptoms are paresthesia, dysarthria, visual loss, deafness, and ataxia (9-11). Ataxia is one of the neurodegenerative symptoms that characterized by unbalance disorders and cause a decrease in quality of life in patients. Therefore, the early detection of its symptom particularly in the community with high risk exposed by low concentration of MeHg can prevent the more severe related symptoms in the future. The purpose

of this study is to investigate the effect of low dose exposure of MeHg in cerebellar dysfunction detected by the present of ataxia and Purkinje cells feature in MeHg-induced Balb/c mice.

MATERIALS AND METHODS

Sample and treatment

This study was an experimental research with a post-test only control group design, used 15 healthy male Balb/c mice, with a weight of 24-25 grams and age of around 12 weeks. They were randomly divided into 3 groups and treated for 17 days with the following treatments such as control (saline solution), MeHg1 (a low dose MeHg solution at 5 mg/kg BW) and MeHg2 (a high dose MeHg solution at 10 mg/kg BW) group. MeHg solution was prepared by dissolving MeHg and L-cysteine powder with a saline solution. The body weight of mice was daily measured and MeHg solution volume was adjusted according to mice body weight. The treatment was orally administered. Mice were acclimatized 1 week before the treatment.

Detection of ataxia

The grade of ataxia was observed every day using the Hind-limb Clasping test by observing the clasping sign of mice hind and limb for about 10 seconds, as well as Ledge test by checking the balance during walking on cage's ledge. Then, the results of the ataxia examinations by the Hind-limb Clasping and Ledge test were divided in to 4 scores; which were score 0 or no ataxia (all feet consistently stretched out from abdomen, or mice could walk balance along the cage edge and back into the cage smoothly); score 1 or mild ataxia (one legs pulled toward the abdomen for more than 5 seconds, or slipped his footing when walking along the cage edge but still has a coordination on inter-step movements); score 2 or moderate ataxia (two legs partially pulled toward the abdomen for more than 5 seconds, or could not use feet effectively but can still walk on the cage edge and lowering itself by falling the heads rather than the claws); and score 3 or severe ataxia (all legs were totally pulled and touched the abdomen for more than 5 seconds, or falling from the cage edge and unable to walk by itself) (9). There were 2 mice on MeHg groups that experienced severe ataxia with severe malaise and excessive weight loss (more than 5 grams per day), therefore they were terminated earlier at days 11 and 15 for MeHg2 and MeHg1 group, respectively. These mice were included in the data analysis.

Histopathological examination

After termination, the brain was separated for cerebrum (right and left) and cerebellum part. Each part has measured the weight and then stored at the tube with a 10% formalin buffer solution. Cerebellum tissue was undergone histopathological staining with Hematoxylin Eosin (HE) and examined the number of intact Purkinje cells by the Pathologist with a blinded sample. Intact Purkinje cells are appeared rounded, intact and have a nucleus, while damaged cells are shown by cells atrophy, disappeared nucleus, and irregular shape. The interpretation of the histopathological slide was obtained from the average of intact Purkinje cells number from 10 fields of view with 400x magnification.

Data analysis

The result of normality test by Shapiro-Wilk test indicated that the data was not normally distributed ($p < 0,05$; data was not shown). Then, the association of ataxia level among treatment groups was checked by using the Fisher Exact test. The effect of MeHg administration on brain histopathology was measured using the One-Way ANOVA test and then followed by Post-Hoc tests. The relationship between the degree of ataxia and brain histopathological change was checked by a Kruskal-Wallis test. The level of significant was suggested by $p < 0.05$.

RESULTS

Effect of MeHg treatment in ataxia

The distribution of ataxia is shown in Table I. There is a significant difference in ataxia among the group ($p < 0.001$; Fisher Exact test). The degree of ataxia was different among groups, where the MeHg2 group (10 mg/kg BW/day) has the earliest and most severe ataxia compare with other groups. There was no ataxia observed in the control group.

Table I : The correlation between the degree of ataxia and MeHg administration

Groups	Ataxia disorder; n (%)			*p*
	No ataxia	Mild	Severe	
Control	5 (100)	0	0	
MeHg1	0	4 (80)	1 (20)	<0.001*
MeHg2	0	0	5 (100)	

A comparison among the group used Fisher's Exact test. *Significance value when $p < 0.05$

Effect of MeHg treatment in brain histopathological features

The feature of brain histopathology was shown as the different of intact Purkinje cell number in cerebellum tissue. As shown in Table II, there was a significant difference in intact Purkinje cell numbers among different treatment groups ($p = 0.001$). The lowest number of Purkinje cells number was shown in MeHg2 group (at dose 10 mg/kg BW) and significantly difference when compared with MeHg1 (at dose 5 mg/kg BW) and normal control (7,65±0,59 vs 6,26±1,17 and 5,12±0,38; both $p < 0.05$). There was also a significant difference of intact Purkinje cell number between MeHg1 and normal control group ($p = 0.016$; Post-Hoc test; see Figure 1).

In the control group (Figure 2.a), the distribution of Purkinje cells was observed between the molecular (right) and granular layers (left). Normal Purkinje cells are appeared in normal control group (Figure 2.b),

while damaged cells are shown in MeHg treated groups (Figures 2.c and 2.d).

Table II : The brain histopathological features of MeHg-induced brain Balb/c mice

Groups	Purkinje cells number	*p*
	Mean ± SD	
Control	7,65 ± 0,59	
MeHg1	6,26 ± 1,17	0.001*
MeHg2	5,12 ± 0,38	

The difference number of Purkinje cells number among groups was analyzed using a One-Way ANOVA test. *Significance value when $p < 0.05$

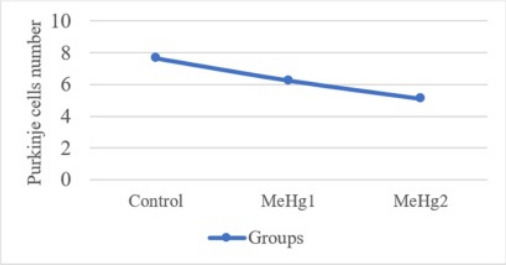

Figure 1 : The difference of Purkinje cells number between treatment groups. P-value for control vs MeHg1 and MeHg2 group was 0.016 and 0.001, respectively, and MeHg1 vs MeHg2 was 0.041 (Post-Hoc test).

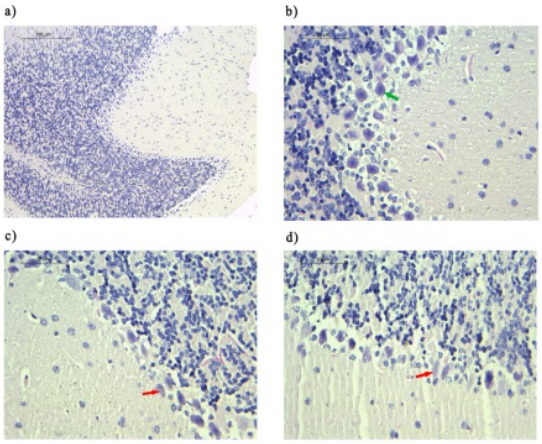

Figure 2 : Microscopic features of the cerebellum Balb/c mice in control (a and b), MeHg 5 mg/kg/BW (c), and MeHg 10 mg/kg/BW (d). Fig (a) was at 100x magnification and (b), (c), (d) were at 400x magnification. The analysis by the pathologist was done for 10 view fields.

The relationship between ataxia and histopathological features

Table III shows the intact Purkinje cells number was reduced according to the severity of ataxia and has a significant difference among groups of no-ataxia, mild and severe ataxia in Balb/c mice ($p < 0.05$; Kruskal-Wallis test).

Table III : An overview of ataxia with histopathological features of brain

Ataxia disorder	Purkinje cells number	*p*
	Mean ± SD	
No ataxia	7.65 ± 0,68	
Mild	7.25 ± 0.07	0.014*
Severe	5.05 ± 0.35	

The difference number of Purkinje cells number among ataxia disorder groups was analyzed using a Kruskal-Wallis test. *Significance value when $p < 0.05$

DISCUSSION

The results of this study showed a similar effect of MeHg in inducing ataxia, even though at low dose of exposure. Previous study reported six out of seven mice that were treated using MeHg showed the presence of hind-limb clasping at the end period of the research (12). Other studies indicated that MeHg intoxication was correlated with ataxia (4, 9, 16). Mercury compounds that enter the body can be distributed into several tissues particularly liver, kidneys, and brain (13). MeHg is a lipophilic compound, which can easily penetrate the lipid membrane including the blood-brain barrier (BBB) and placental barrier (13). The binding of MeHg to cysteine in red blood cell hemoglobin will make a MeHg-cysteine complex, that acts as an amino acid analogue and has a similar structure to the essential amino acid methionine therefore it can be transported by the L-type large neutral amino acid transporter-1 (LAT-1 to pass through BBB and cause brain damage (14, 15). The result of this study showed that the low dose of MeHg could induce the cerebellar lesions by reducing intact Purkinje cells number as causes of ataxia.

This study found a significant relationship between MeHg treatment and ataxia in Balb/c mice in a dose-dependent manner. The Ledge test and Hind-Limb Clasping test that was used to examine the presence of ataxia indicated that mice-treated with higher dose of MeHg (10 mg/kg BW) had more severe ataxia compared with lower dose (5 mg/kg BW), while it was not found in all of the negative control mice. This study's result showed that MeHg even thought at a low concentration could also cause ataxia at mild stage, indicating that ataxia could be used as one of the early symptoms to detect MeHg intoxication.

One of the MeHg toxicity mechanisms is through mitochondrial damage by formation of free radicals and reduction of Glutathione (GSH), an intracellular tripeptide that plays in the process of detoxification, antioxidants, and cell proliferation. The binding of MeHg and thiol compounds can reduce the amount of sulfhydryl and GSH which play an important role in reducing the impact of oxidative damage (17). It affects increasing reactive oxygen stress (ROS) particularly hydrogen peroxide (H_2O_2) (18, 19). MeHg exposure also causes on inducing pro-inflammatory cytokine

expressions in the brain cells and macrophages, such as IL-6, IL-8, MCP-1, MIP-2 and MCP-5 (19-21). Mitochondria function is related to apoptosis mechanisms, such as a phosphatidylserine transfer to the outside of the mitochondrial membrane which is a trigger factor for cell death. The influence of MeHg-induced free radicals formation in cerebellum could affect the form and number of intact Purkinje cells due to atrophy and the process of apoptosis (22). In this study, the histopathological feature of intact Purkinje cells number in the cerebellum was significantly different between the MeHg-treated and control group, where mice treated with a high concentration of MeHg had the lowest number of intact Purkinje cells compared with lo concentration and negative control mice. It indicates that the amount of MeHg in the brain is related to the histopathological features change in the brain of Balb/c mice, particularly in the number of intact Purkinje cells.

Ataxia can arise as a result of idiodendritic branch atrophy of Purkinje cells that cause a decreasing intact cell number (23). Dendritic fibers from Purkinje cells synapse with granular, stellate, and inferior olivary nuclei cells, making the Purkinje cell receives more than 100,000 inputs and becoming the only output cell in the cerebellar cortex (24). Since all cells are GABAergic, the entire output of Purkinje cells is inhibitory which has a function to modulate excitation. Previous studies reported that MeHg could induce inflammation in the brain by activating numerous pro-inflammatory cytokines expression and generating stress oxidative (19, 25-30). The atrophy of Purkinje cells and dendritic fibers can affect the process of impulse distribution and synaptic cells, then causing clinical symptoms such as motor disorders and ataxia (23). Ataxia can also be caused by disruption of impulse distribution in the cerebellar cortex, dentate nucleus, inferior olivary nucleus, atrophy of the pons, glial cell disorders, atrophy of the cerebellar cortex, and cerebello-olivarius atrophy (23). Therefore, the onset of ataxia can be affected by a decrease in the number of Purkinje cells in the cerebellum Balb/c mice, but the degree of ataxia can be influenced by several components in the brain other than Purkinje cells, which can cause different degrees of severity. Our previous study also reported the relationship between ataxia and intact Purkinje cells number (16), however the onset and severity of ataxia that related with Purkinje cells damaged and different doses of MeHg exposure was not yet investigated. This study showed that ataxia has shown at early onset and more severe on the MeHg highly dose treated group, indicating that higher MeHg amount entered and accumulated in the cerebellum could induce earlier and more severe of Purkinje cells damage that related with ataxia severity. However, the involvement of dendritic fibers and other related biomarkers such as GABA is not yet investigated in this study.

CONCLUSION

MeHg exposure at low concentration has a significant impact on reducing intact Purkinje cells number in the cerebellum and that was related to severity of ataxia. It indicates that ataxia appearance could be used as an early sign to detect MeHg intoxication in the exposed population. The further studies on the involvement of dendritic fibers and other biomarkers that are related with ataxia such as GABA are needed to know the detail mechanism of MeeHg-induced ataxia.

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