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Membranes and deoxyribonucleic acid of hippocampal neurons damage due to low-density polyethylene microplastics in blood of Wistar rats

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***Abstract*---**Microplastic particles in the blood can cause damage to organs such as the brain. This study aimed to analyze the effect of microplastic particles in the blood on membrane damage (expression of malondialdehyde metabolites) and deoxyribonucleic acid damage (expression of 8-hydroxy-2′-deoxyguanosine metabolites) in hippocampus

neurons of Wistar rats. Methods: Forty-two Wistar rats were used and equally divided into six groups. The study groups X1, X2, X3, X4 and X5 was given 0.0375mg, 0.075mg, 0.15mg, 0.3mg, and 0.6mg of low-density polyethylene microplastic powder mixed with 2cc distilled water respectively, while the control group only given distilled water. Microplastic administration was carried out for 90 days. Results: Microplastic particles were found in the blood of Wistar rats. The level of microplastics particle was higher along with the higher exposure dose. The mean expression of malondialdehyde and 8-hydroxy-2′-deoxyguanosine metabolites in the hippocampal neurons in CA1 and CA3 areas were significantly increased with higher exposure doses (Kruskal-Wallis test $p < 0.01$). The Spearmen's correlation showed a strong relationship between the levels of microplastic particles in blood and the expression of metabolites malondialdehyde and 8-hydroxy-2′-deoxyguanosine (all $p < 0.01$). Microplastic in the blood of Wistar rats has increased expression of malondialdehyde and 8-hydroxy-2′-deoxyguanosine metabolites in hippocampal neurons.

***Keywords*---**8-hydroxy-2′-deoxyguanosine, Environmental pollution, Hippocampal neurons damage, Malondialdehyde, Microplastics.

Introduction

Microplastics (MPs <5mm) have become pollutants in the human food chain (Cox KD et al, 2019; Jiang S et al, 2021). This is evidenced by the discovery of plastic fibre particles accumulating in the organs of living things (Karbalaei S, Hanachi P, Walker TR, Cole M, 2018). Human faeces contain MPs polymeric particles of polyethylene, polystyrene, polyvinyl chloride and polyethylene terephthalate were reported (Schwabl P et al, 2019; Yan Z et al, 2019). The entry route for MPs in humans is not only through contaminated food, but also through inhalation of polluted air and skin contact with MPs. MPs with a diameter of ~20µm will circulate in the blood 1-2 days post ingestion for distribution to all organs and tissues of the body (Wright SL, Kelly FJ, 2017). MPs with a diameter of ≤10µm can penetrate the blood brain barrier by passive transport because they have a fat-soluble composition of hydrophobic organic contaminants [7]. Whole component of MPs will trigger biological responses, such as inflammation, genotoxicity, oxidative stress, hypersensitivity, and unwanted immune responses that end in cell death (Karbalaei S et al, 2018; Companale C et al, 2020; Hwang J et al, 2019).

In body tissue, MPs are persistent, bio-accumulative, and toxic due to the free radicals formed (Deng Y et al, 2017; Shengchen W et al, 2021). In the brain, it causes neurodegeneration of neurons. The dose, particle size and length of exposure time determine the effects of brain damage and disease (Dehaut A et al, 2016). Normally, free radicals are present in every living thing. However, high levels cause cellular damage via reactive oxygen species (ROS) formation (Pomatto LCD et al, 2018). Damage occurs because cellular antioxidant levels are lower than oxidant levels (Trestrail C et al, 2020). Various studies have mentioned low antioxidant levels in animal exposed to MPs (Karbalaei S, 2018; Companale C, 2020; Trestrail C et al, 2020). The number of neurons damage

causes disruption of cognitive abilities as the main function of the brain. The hippocampus is one of the anatomical structures that play a role in cognitive function. There are 4 areas called the Cornu Ammonis (CA), namely CA1, CA2, CA3 and CA4. The learning function is played by the CA1 area and the learning function in the CA3 area [14,15] (Cao Y et al, 2016; Konen LM et al, 2020).

Cellular death can be through membrane or deoxyribonucleic acid of neurons damage. Free radicals in the form of hydroxyl radical (OH*) and hydrogen peroxide (H2O2) formed from the Haber-Weiss and Fenton reaction play a role in damaging membrane and deoxyribonucleic acid neurons (Xu TANZ et al, 2019; Ribeiro IML et al, 2020). Cellular metabolite of phospholipid membrane damage is malondialdehyde (MDA), while deoxyribonucleic acid damage is 8-oxo-7,8-dihydro-2-deoxyguanosine (8-OHdG) (Song P et al, 2021). These cellular metabolites can be observed under a microscope by immunohistochemical staining. The presence of these cellular metabolites indicates neurons damage. Another important point in this study is about an adequate dose of MPs that can cause neurons damage in the hippocampus

Wistar rats were used as a model to assess the presence of cell damage in hippocampal neurons due to the administration of toxic substances (microplastics). Wistar rats are genetically homologous to humans so the results of this study can be used as the basis for further research on higher mammalian orders. The hippocampus of Wistar rats is structurally different from humans but is functionally similar (Golcaves EN et al, 2018).

Therefore, the aim of this study was to analyze low-density polyethylene microplastic particles in the blood that cause membrane and deoxyribonucleic acid damage in hippocampal neurons of Wistar rats based on observed expression of malondialdehyde (MDA) and 8-hydroxy-2′-deoxyguanosine (8-OHdG) metabolites in hippocampal neurons.

Method

Experimental research design

This was an experimental laboratory study with a post-test only control group design to examine the effects of blood microplastic particles on the hippocampus neurons. The research was carried out at the Biomedical Laboratory Widya Mandala Surabaya Catholic University and Research Centre Universitas Airlangga, Indonesia. The research was conducted from October 2021 until March 2022. Lemeshow formula at α 0.05 and β 0.05, obtained 7 Wistar rats for each group. Forty-two (N =42) Wistar rats were divided into six groups by random allocation. Wistar rats were obtained from Farma Veterinary Center Surabaya and had a certificate of eligibility as experimental animals. Wistar rats were male, 12 weeks old, weighing 160-175 grams, fed and watered at libitum, clean care environment, stable air circulation, room temperature 18-26ºC, and humidity 40-70%. Each Wistar rat in the experimental group was given of dry low-density polyethylene microplastics powder per day dissolved in 2cc of distilled water through an oral probe. Study group 1 (X1) was given 0.0375mg, study group 2 (X2) was given 0.075mg, study group 3 (X3) was given 0.15mg, study group 4 (X4) was given

0.3mg, and study group 5 (X5) given 0.6 mg of microplastic per day. While the control group (K) only given distilled water without microplastics. After 90 days, there was no drop out, Wistar rats were anesthetized and 1cc of blood was taken through cardiac puncture to measure the number of microplastic particles in the blood. After the blood collection procedure was completed, Wistar rats were euthanized with cervical dislocation technique until vital signs of life such as heart rate were not detected and pupillary reflexes were negative. Wistar rats were dissected through the head and removed the brain. After making histopathological preparations in the hippocampus area, anti-MDA and 8-OHdG immunohistochemical staining were made. Results of the microscopic examination were statistically analyzed to assess the effect of microplastic administration on the levels of microplastic particles in the blood of Wistar rats. After that, we analyzed the correlation between the level of microplastic particles in blood and the expression of malondialdehyde (MDA) and 8-hydroxy-2′-deoxyguanosine (8-OHdG) metabolites in hippocampal neurons. Adequate doses of microplastics in the blood that can damage hippocampal neurons were also be analyzed using statistical tests.

Microplastics processing

Microplastic (MPs) particles were made from Low-Density Polyethylene (plastic wrap) which was fragmented using Miller FCT-Z100 (Fomac, Indonesia). Milling until the size of MPs particle is fine. The plastic powder filtered with an 800mesh sieve (Anping Tianhao Wire Mesh Products Co., Ltd, China) to produce particles measuring <20μm.

Assessment of blood microplastics levels

The procedure for examining blood levels of MPs refers to the method previously described by Monteleone et al (2019) namely destruction of the blood, isolation of microplastic particles, and examination of blood microplastic particles level. Wistar rat blood samples were prepared at Biomedical Laboratory of Widya Mandala Surabaya Catholic University. The level of MPs particles in blood was calculated by clinical pathologist at Neuron Clinic Laboratory in Surabaya using binocular light microscope.

Immunohistochemical staining procedures

Examination of malondialdehyde (MDA) metabolites expression in hippocampal neurons following the manufacturer's protocol for JAI-MMD-030N anti-Malondialdehyde [MDA] mAb (1F83) (AdipoGen Life Sciences, Inc® San Diego). This protocol was used and described previously by Li et al, 2013. Examination of the expression of 8-hydroxy-2′-deoxyguanosine (8-OHdG) metabolites in hippocampal neurons followed the manufacturer's protocol for 8-OHdG monoclonal antibody (Immundiagnostik AG® Germany). This protocol was used and previously described by Topal et al, 2017. This immunohistochemical preparation procedure was carried out at the Anatomical Pathology Laboratory, Universitas Airlangga.

Assessment of immunohistochemical slide

Immunohistochemical assessment of hippocampal neurons of Wistar rats was performed by an anatomic pathologist using binocular light microscope at 40x and 400x magnification. Calculations of the mean number of hippocampal neurons expressing malondialdehyde (MDA) and 8-hydroxy-2′-deoxyguanosine (8-OHdG) metabolites was performed in Cornu Ammonis 1 (CA1) and Cornu Ammonis 3 (CA3) areas in each of five fields of view. The expression of metabolites MDA and 8-OHdG showed dark brown cells (red arrows on figure) due to the use of the DAB chromogen. The brown precipitate in these cells will not dissolve in alcohol and xylene during the rehydration process.

Research data analysis

The results in this study were presented in the form of a graph of the mean ($\bar{x}$) ± standard deviation (SD) and immunohistochemical microscopic images of the expression of malondialdehyde (MDA) and 8-hydroxy-2′-deoxyguanosine (8-OHdG) metabolites in hippocampal neurons. Normality test was performed using the Shapiro-Wilk test. The test showed a group with p value <0.05 which indicate that the research data was not normally distributed. Therefore, the statistical analysis was performed by nonparametric test. Kruskal-Wallis test was used to determine the significance of differences in the number of MPs particle in blood, expression of malondialdehyde neurons, and expression of 8-hydroxy-2′-deoxyguanosine neurons between groups at 95% confidence intervals. The correlation between the number of MPs particle in the blood and the expression of malondialdehyde (MDA) and 8-hydroxy-2′-deoxyguanosine (8-OHdG) metabolites in hippocampal neurons used Spearmen's Rho test. Multiple comparison tests were used to determine the adequate dose that caused damage to membrane and deoxyribonucleic acid of hippocampal neurons. The Mann-Whitney U test was used as a multiple comparison.

Ethics statement

Research ethics approval was obtained from the Health Research Ethics Committee (HREC) Widya Mandala Surabaya Catholic University with a certificate number 209/WM12/KEPK/DOSEN/T/2021.

Discussion

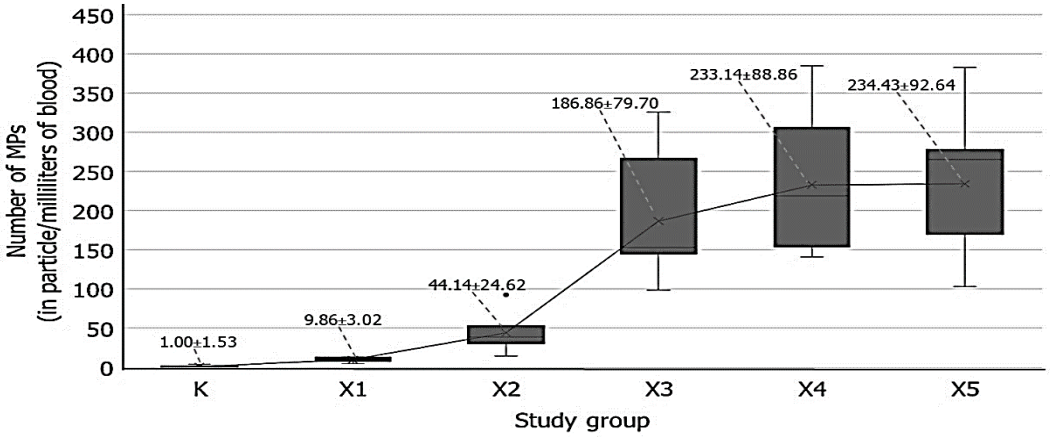


Figure 1. Mean ± SD levels of blood microplastic particles

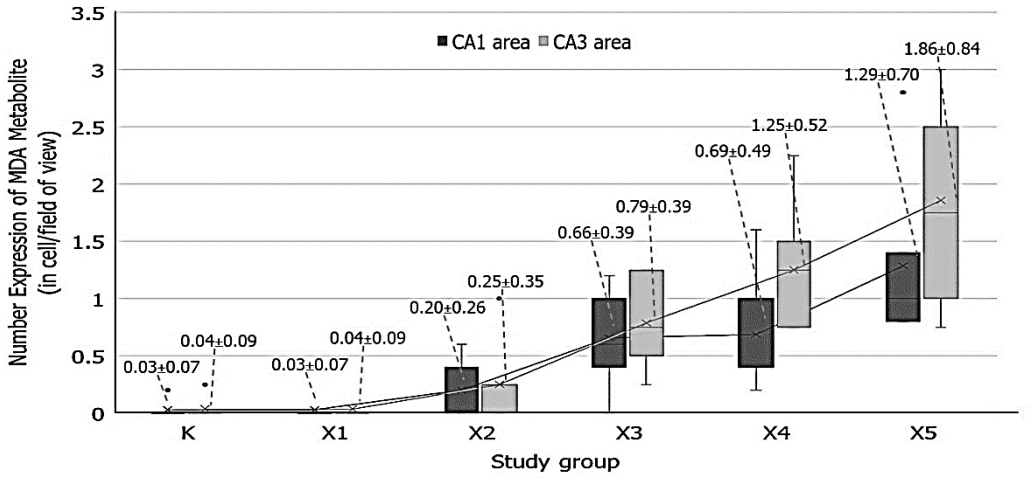


Figure 2. Mean ± SD expression of malondialdehyde metabolites in hippocampal neurons

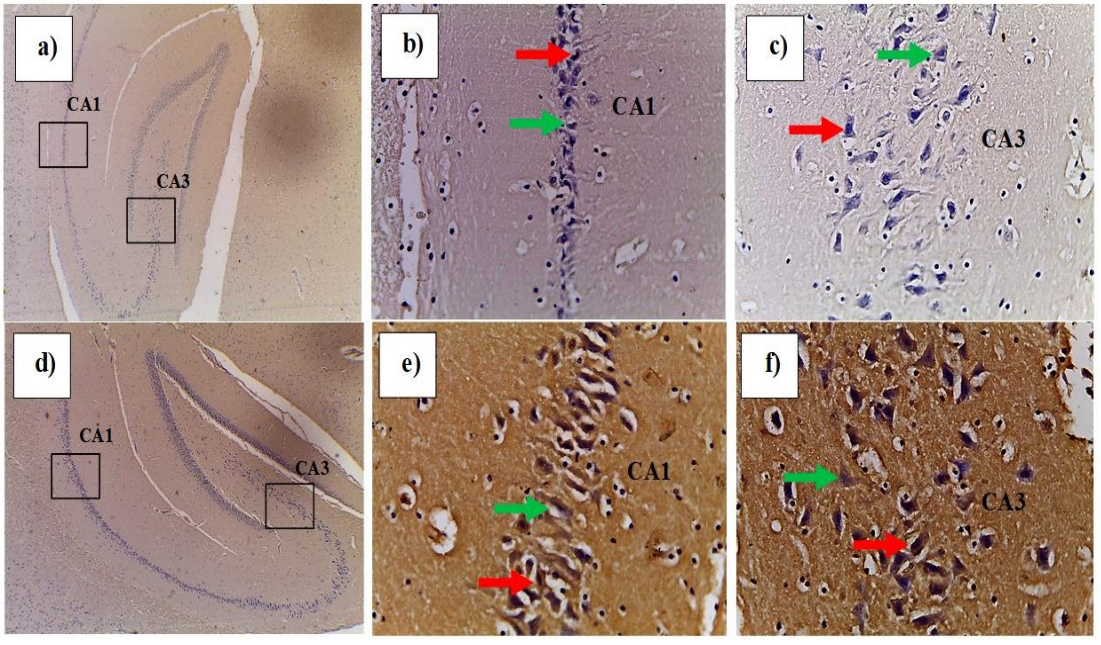


Figure 3. Histopathological images of hippocampal neuron membrane damage in Wistar rats, stained with immunohistochemical. Description: a) control group with CA1 and CA3 area, 40x magnification; b-c) MDA expression in CA1 and CA3 area, unexposed to MPs, microscope 400x magnification; d) study group with CA1 and CA3 area, 40x magnification; e-f) MDA expression in CA1 and CA3 area, exposed to MPs, microscope 400x magnification; red arrows is hippocampal neurons expressing MDA; green arrows is normal cell neuron.

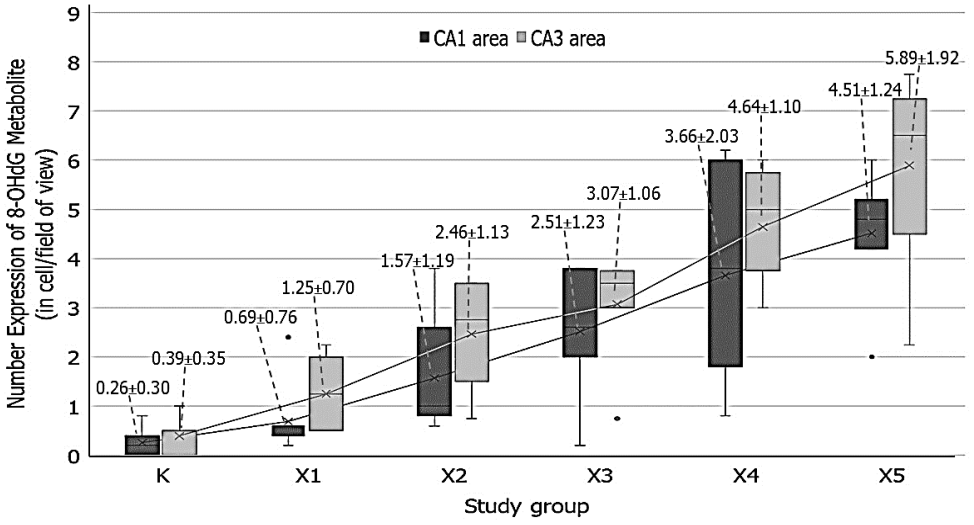
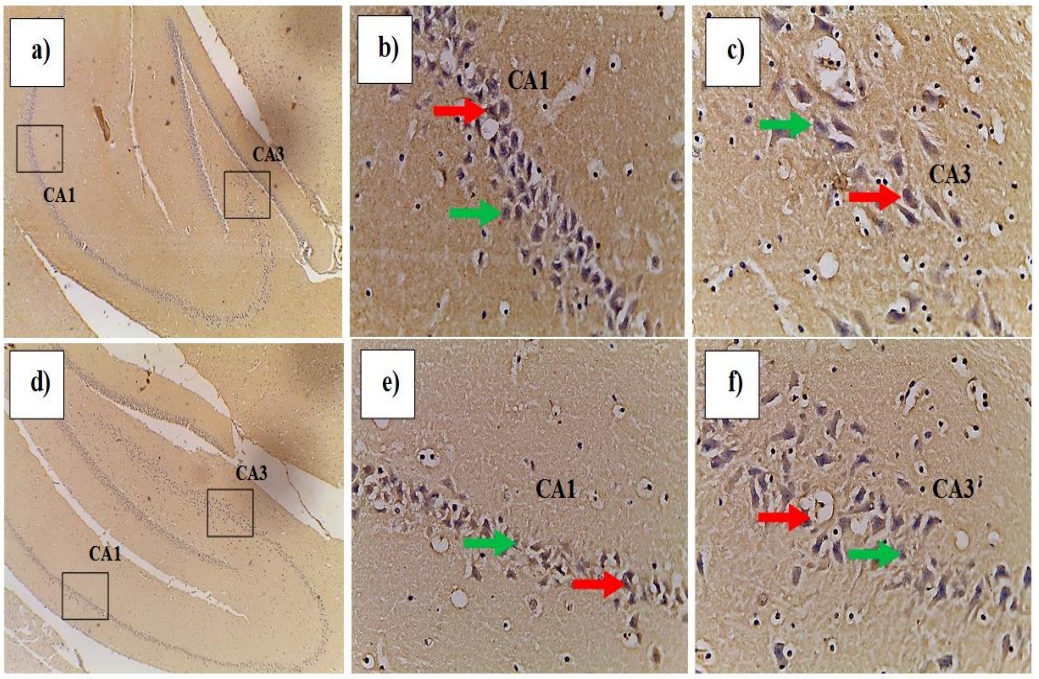


Figure 4. Mean ± SD expression of 8-hydroxy-2′-deoxyguanosine metabolites in hippocampal neurons



group with CA1 and CA3 area, 40x magnification; b-c) 8-OHdG expression in CA1 and CA3 area, unexposed to MPs, microscope 400x magnification; d) study group with CA1 and CA3 area, 40x magnification; e-f) 8-OHdG expression in CA1 and CA3 area, exposed to MPs, microscope 400x magnification; red arrows is hippocampal neurons expressing 8-OHdG; green arrows is normal cell neuron.

Table 1
Comparison of MPs particle in blood, expression of MDA and 8-OHdG metabolite in hippocampal neurons between groups

Variable	p value of Kruskal-Wallis statistical test
MPs particle in blood	<0.01
MDA metabolite expression at CA1	<0.01
MDA metabolite expression at CA3	<0.01
8-OHdG metabolite expression at CA1	<0.01
8-OHdG metabolite expression at CA3	<0.01

Table 2
The correlations between number of MPs particle in blood with the expression of MDA and 8-OHdG metabolites

Parameter	Expression of MDA Metabolites	Expression of 8-OHdG Metabolites
Correlation Coefficient	.755**	.842**

p value of Spearman's rho	<0.01	<0.01

Table 3
Multiple comparison analysis between groups on MDA and 8-OHdG metabolites expression variable in hippocampal neurons

Group	p value of Mann-Whitney U test on MDA metabolites expression						p value of Mann-Whitney U test on 8-OHdG metabolites expression					
	K	X1	X2	X3	X4	X5	K	X1	X2	X3	X4	X5
K	-	1.000	0.155	0.001	0.001	0.001	-	0.059	0.003	0.004	0.002	0.002
X1	1.000	-	0.155	0.001	0.001	0.001	0.059	-	0.040	0.017	0.003	0.003
X2	0.155	0.155	-	0.020	0.006	0.002	0.003	0.040	-	0.222	0.021	0.004
X3	0.001	0.001	0.020	-	0.333	0.010	0.004	0.017	0.222	-	0.140	0.017
X4	0.001	0.001	0.006	0.333	-	0.054	0.002	0.003	0.021	0.140	-	0.158
X5	0.001	0.001	0.002	0.010	0.054	-	0.002	0.003	0.004	0.017	0.158	-

We all already know that plastic pollutants have polluted sea water, fresh water and soil ecosystems globally (Walker TR, 2019). Currently, small size of plastic particles was reported to contaminate human food and drink (Cox KD et al, 2019; Jiang S et al, 2021). One of them is low-density polyethylene (LDPE). This will be a serious problem in the future, especially in the health sector (Jiang S et al, 2021; Jambeck JR et al, 2015; Miraj SS et al, 202). In this study, LDPE was formed into microplastic (MPs) particles. These microplastics are <20μm in diameter with sharp and irregular edges. The shape of MPs determines the biological response (Wright SL, Kelly FJ, 2017).

The findings of this study indicate that MPs particles have been found in blood of Wistar rats given orally MPs for 90 days. The number of MPs particles found in the blood was higher in the Wistar rats group given higher doses of MPs (Figure 1). Previous research stated that MPs particles with diameter ≤20μm in the digestive tract can enter the blood circulation system through endocytosis mechanism, then can be distributed to various organs in the body (Lei L et al, 2018; Wu B et al, 2019; wang YL et al, 2019). At sizes <10 μm, MPs particles can pass through cell membranes and are found in the placenta, muscle, and brain (Campanale C et al, 2020). Administration of higher MPs doses will cause more contact between MPs particles and the surface of microfold cells at the tips of the intestinal villi, thereby increasing the potential for MPs particle endocytosis. These results are similar to the findings of (Hamed et al, 2019), in Tilapia was given 1mg/L, 10mg/L, and 100mg/L of MPs for 15 days. They stated that the number of MPs particle per gram of fish tissue increased along with higher exposure doses. In their study, it was not known how many doses of MPs particle were consumed by Tilapia, but our study explained between the MPs exposure dose and the number of MPs particle found in the blood.

The current study found that the MPs particles level in blood is significantly and positively correlated with the neuron damage in the Wistar rats. The findings of this study indicate the occurrence of membrane (expression of malondialdehyde metabolite) and deoxyribonucleic acid (expression of 8-hydroxy-2′-deoxyguanosine metabolite) damage in hippocampal neurons of Wistar rats due to low-density polyethylene microplastics. This damage can occur because MPs particles in the blood circulation or accumulated in the organ tissues cannot be destroyed by phagocytic cells. Even so, cellular defence continue to destroy them through oxygen-dependent and oxygen-independent mechanisms. Oxygen-dependent mechanisms such as oxidative stress, while oxygen-independent mechanisms such as the role of inflammatory mediators (Othman AR et al, 2021; Li Z et al 2020). The final response of this mechanism will induce cell damage (Hwang J et al, 2019; Trestrail C et al, 2020).

Malondialdehyde (MDA) as an indirect index of oxidative damage caused by lipid peroxidation in hippocampal neuronal membranes. Deoxyguanosine (dG) is one of the bases that compose the deoxyribonucleic acid of hippocampal neurons, which when oxidized will become 8-hydroxy-2'-deoxyguanosine (8-OHdG) (Song P, 2021). Initially, microplastics consumed will increase intracellular antioxidant enzymes such as superoxide dismutase and catalase. However, if microplastics continue to enter the body, these enzymes will decrease because they are unable to reduce the free radicals that are formed. This has been proven by many studies with exposure to MPs (Trestrail C et al, 2020; Li Z et al, 2020; Sokmen TO et al, 2020). In this condition, cell damage begins to occur, such as an increase in the expression of MDA and 8-OHdG metabolites as an indicator of free radical reactions in experimental animals (Shengchen W et al, 2021).

This finding is in line with the study by Li et al, 2020, in Wistar rats fed 5mg/L of polystyrene MPs for 90 days (≈ 0.15mg of dry MPs particles per day) showed that myocardium damage was triggered by oxidative stress. Study by Sokmen et al (2020), stated that similar to the findings of this study, oxidative stress occurred in zebrafish embryo brain tissue exposed to 20nm diameter polystyrene MPs for 120 hours and increased expression of the 8-OHdG metabolites. Study by Cheng et al (2020), which used LDPE MPs with a diameter of 550-1000μm for 28 days showed an increase in oxidative stress which led to increased expression of MDA and 8-OHdG metabolites genes in Eisenia Fetida. Similarly, in the study by Chen et al (2020), in Eisenia Fetida exposed to LDPE MPs with a diameter <400μm at a minimum dose of 1 g/kg dry powder showed an increase in MDA metabolites in intestinal tissue.

The toxicity of LDPE MPs comes from plastic particles that cannot be destroyed through cellular defense mechanisms. In addition, it also comes from toxic compounds contained in these particles due to the production process or absorbed when low-density polyethylene is in nature (Wright SL, Kelly FJ, 2017). The toxic compounds are still found in the form of fragmented plastics (Wright SL, Kelly FJ, 2017; CampanaleC et al, 2020; Brennecke D, 2016).]. When these plastic particles or compounds enter the body, it will trigger the breaking of covalent bonds of atom into unpaired electrons (Trestrail C, 2020). Free radicals are molecules or atoms that have one or more unpaired electrons in their outer

shell so they are highly reactive and radical. This reaction will occur continuously because the MPs particles cannot be destroyed by the body's defense mechanisms. If the concentration of cellular antioxidants is insufficient to neutralize these free radicals, it will cause oxidative stress. Oxidative stress will continue with damage to cell structure. Continuous damage will cause a disease (Pomatto LCD et 2018; Trestrail C, 2020). In the brain, oxidative stress causes neurodegeneration through neurons damage (Xu TANZ et al, 2019; Ribeiro IML, 2020; Song P et al, 2021). Brain neurodegeneration is a pathophysiology of decreased brain function. One of them is cognitive function played by the hippocampus.

Wistar rats hippocampal neurons damage was seen significantly when given MPs at dose of 0.075mg per day for 90 days (equivalent to 45 MPs particles in milliliters of blood). In humans weighing ± 70 kilograms, this MP exposure dose is equivalent to 3.47mg. This means that if humans consume as much as 3.47mg of MP particles every day, there will be significant damage to their hippocampal neurons.

This study has proven that MPs in the blood cause damage to the hippocampal neurons as seen from the expression of MDA and 8-OHdG metabolites in hippocampal neurons. The results of this study cannot be generalized to other neurons in the brain. In addition, this study has limitations such as not examining the presence of MPs particles in the brain of Wistar rats, the size of the MPs particle used varies up to 20µm in diameter, and does not examine the concentration of other toxic compounds contained in the blood of Wistar rats due to MPs exposure. As our recommendation, in other studies it is necessary to measure the levels of MPs particle in the brain (especially the hippocampus) and identify various toxic substances in the blood due to MPs exposure. This substance also has a toxic effect on brain neurons such as phthalates, bisphenol-A, polybrominated diphenyl ether, dichloro-diphenyl-dichloroethylene, dichloro-diphenyl-trichloroethane, polycyclic aromatic hydrocarbons, and polychlorinated biphenyls.

Conclusion

Administration of low-density polyethylene MPs <20µm in diameter for 90 days caused the presence of microplastic particles in the blood of Wistar rats. The higher number of MPs particles in the blood causes an increase in the expression of MDA and 8-OHdG metabolites in the hippocampal neurons. These expressions indicate the hippocampal neurons damage in membranes and deoxyribonucleic acid.

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