Liver Abnormalities in Wistar Rats Exposed to Oral Intake of Polyvinyl Chloride Microplastics

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Abstract

Microplastics is a food contaminant. Smaller microplastics can penetrate into the blood circulation system and bioaccumulate in the liver. It can induce oxidative stress in hepatocytes, resulting in damage. The aim of this experimental study was to compare the levels of functional biomarkers, anatomical, and histopathological features of the liver due to oral microplastics exposure. Fourteen white rats were used and equally divided into two groups. The experimental group (E) was given 0,5mg polyvinyl chloridemicroplastics dissolved in 1cc of distillated water per day, while the control group (C) was only given distillated water. Both groups were given orally for 28 days using a probe. Therewasno difference in blood level of serum glutamic oxaloacetic transaminase, serum glutamic pyruvic transaminase, lactate dehydrogenase, tryglicerides, and total cholesterol between both groups. The anatomical features indicated a normal condition. Although it was not significant, experimental group's liver weight (9.6±1,6grams) tended to be heavier compared to control group (8.7±1,4grams). The histopathological hallmarks of hepatocellular injury were more noticeable in experimental group, either a reversible process such as hepatocytes degeneration (C=119,5±34,6; E=186,7±11,5) or cell death (C=13,7±5,1; E=38,5±12,7). Statistical test showed very strong significance (p <0,01). Lobular inflammation and Kupffer cells were also more prominent. Oral intake of polyvinyl chloridemicroplastics in white rats causes hepatocellular injury, specifically hepatocyte degeneration. However, the dose of microplastics used was not sufficient to alter gross anatomy and biomarkers of liver function in blood.

Keywords: hepatocellular injury; hepatotoxicity; liver cell death; microplastics; polyvinyl chloride

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Introduction

Asia is the largest plastic producer (50%) globally,

followed by Europe (19%), and North America (18%). Indonesia occupies the 2nd position after China as a country that contributes to plastic waste. Plastic is popular because it is cheap, waterproof, and durable(Prokić *et al.*, 2019). Plastic waste in Indonesia reaches 3.22 million metric tons per year(Widianarko and Hantoro, 2018). This figure will continue to increase every time the community's demand for plastic increases(Widianarko and Hantoro, 2018; Prokić *et al.*, 2019).

Its unbreakable nature causes the plastic to be degraded to a smaller size due to the thermal and mechanical oxidation of ultraviolet light. Plastic degradation products measuring less than 5 millimeters are called microplastics(Jambeck *et al.*, 2015; Lusher*et al*., 2017).Microplastics are a concern for researchers because they have a major impact on the environment and living things(Wright and Kelly, 2017). The small size and large number of plastics make it easy for plastics to be eaten and enter the body of marine biota, causing these pollutants to enter the aquatic food chain. Marine biota found containing microplastics are marine fish, shrimp, and shellfish. Other living things that consume marine biota indirectly also comsume microplastics (Widianarko and Hantoro, 2018). Sooner or later the entire food chain will be contaminated with microplastics, and it needs special attention (Lusher*et al*., 2017; Wright and Kelly, 2017; Campanale *et al.*, 2020).

The entry point for microplastics in humans is through contaminated food (Wright and Kelly, 2017).It is found as much as 0.44 particles/gram in sugar, 0.11 particles/gram in salt, 0.03 particles/gram in alcohol, and 0.09 particles/gram in bottled drinking water. Humans are estimated to consume 80 microplastic particles per day through contaminated fruit and vegetables (Campanale *et al.*, 2020). The toxic effects of microplastics on living things are related to the presence of chemicals from the environment attached to microplastics, and additives added to the production process, such as plasticizers, UV stabilizers, flame-retardants, lubricants, and dyes (Wright and Kelly, 2017).The toxicity of microplastics depends on the dose, duration of exposure, and the profile of the exposed subject (Wright and Kelly, 2017; Wu *et al.*, 2019).

The absorption of microplastics in the lumen of the gastrointestinal tract occurs through paracellular persorption and phagocytosis, therefor plastic particles are able to pass through the single layered epithelial gap to enter the circulatory system and be distributed to secondary tissues, such as liver, muscle, and brain(Wright and Kelly, 2017).Microplastics toxicity through mitochondrial induction so that depolarization occurs and inhibits reflux pump activity, adenosis triphospate-binding cassette transporter(Wu *et al.*, 2019).

The cytotoxicity of microplastics to cells and tissues in vivo depends on the size, shape, solubility and surface charge of microplastics. The physical effects of microplastics bio persistence on tissues lead to biological responses such as inflammation, genotoxicity, apoptosis, and necrosis (Wright and Kelly, 2017). In humans, microplastics measuring <50µm are found in the abdominal lymph nodes and <1µm are detected in the liver and spleen. These particles accumulate in cellular macrophages via lymphatic and blood transport. In addition, microplastics interactions in humans can cause hypersensitivity and acute responses such as hemolysis. This is a threat to human health (Campanale *et al.*, 2020).

In marine species, microplastic-associated hydrophobic organic chemicals (HOCs) have been shown to be desorbed into tissues upon ingestion of microplastics. The mechanism of cytotoxicity by microplastic-associated HOCs in humans remains to be studied (Wright and Kelly,

2017). In mammals, identification of high levels of endogenous chemical additives such as phthalate, bisphenol A, polybrominated diphenyl ethers, dichloro-diphenyl-dichloroethylene, dichloro-diphenyl-trichloroethane, polycyclic aromatic hydrocarbons, andpolychlorinated biphenylshas been confirmed. In addition to containing toxic chemicals, microplastics is also a growth medium for harmful pathogenic microbes (Wright and Kelly, 2017). In marine and land animals as well as in humans who consume microplastics, digestive blockages have been reported (Miraj *et al.*, 2021). Polychlorinated biphenylsas part of plastic components can cause neurotoxic, mutagenic, and carcinogenic in the liver (Kumar *et al.*, 2014).

In rats cell, microplastics cytotoxicity induces reactive oxygen species (Hwang *et al.*, 2019).Plastic weathering causes the formation of free radicals through the dissociation of Carbon Hydrogen bonds (Wright and Kelly, 2017).Furthermore, reactive oxygen species induce genotoxic stress and deoxyribonucleic acid damage(Wright and Kelly, 2017; Deng *et al.*, 2018).It is suspected that the accumulation of microplastics is associated with an increase in oxidative stress in the liver. Oxidative stress causes changes in metabolic profile due to disturbances in fat and energy metabolism in the liver (Deng *et al.*, 2017).Increasing the concentration of microplastics causes reduced gene expression followed by decreased levels of antioxidants superoxide dismutase, catalase, and glutathione peroxidase (Li *et al.*, 2020).There are indications that microplastics cause impaired liver lipid metabolism after experimenting with 0.5 and 50grams of microplastics in mice (Lu *et al.*, 2018; Li *et al.*, 2020).Adenosis triphospate, tryglicerides, total cholesterol and lactate dehydrogenase activity in the liver are related to the energy content of the liver.

This study was aimed to analyze changes in functional biomarkers as well as anatomical and histopathological features of the liver due to exposure to microplastics. This research as a theoretical basis for human research, because white rats are genetically homologous with humans.

Materials and Methods

This is an experimental laboratory study with a post-test only control group design to examine the effects of microplastics on the liver. The research was carried out at the Biomedical Laboratory, Widya Mandala Surabaya Catholic University, Indonesia. Twenty (N=14) white rats were divided into control (C) and experimental (E) groups equally by random allocation using a computerized random number generator. The sample size was determined by Lemeshow formula with α=0.05 and β=0.1. The result of the formula is 7 for each group.White rats were male, ± 12 weeks old, weighing 180-200grams, fed and watered at libitum, clean care environment, stable air circulation, room temperature 18-26ºC, and humidity 40-70%. Each white rat in the group E was given 0.5mg of microplastic particles per day dissolved in 1cc of distillated water through an oral probe. While the group C only received distillated water. After 28 days, white rats were anesthetized to draw 2cc of blood through the heart per rat. Furthermore, the white rats were euthanized with cervical dislocation technique until vital signs of life such as heart rate were not detected and pupillary reflexes were negative. The white rats were then dissected through the abdomen and removed the liver.

MicroplasticProcessing

Microplastic particles were made from polyvinyl chloride (PT. SatomoIndovyl Polymer, Indonesia)which was fragmented using Miller FCT-Z100 (Fomac, Indonesia). Milling until the size of microplastics is fine. The plastic powder filtered

with a 625mesh sieve (AnpingTianhao Wire Mesh Products Co., Ltd, China) to produce particles measuring <20µm. Particle size was examined on a microscope Nikon eclipse Ci-L-DS-F12-L3, at 400x magnification and 10 µm scale (Figure 1).

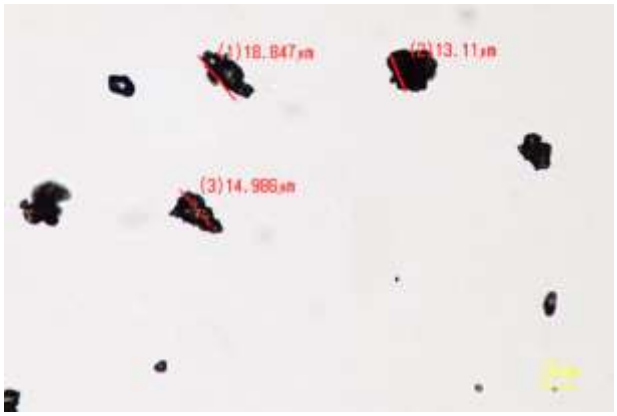

Figure 1. Polyvinyl chloride microplastic size

Liver Biomarker Examination Procedure

White rat blood in plain BD vacutainer was put into a styrofoam box and given blue ice to be taken to the Balai Besar Laboratorium Kesehatan in Surabaya. Delivery takes 30 minute. This method to ensures that the sample does not change and accurate results are obtained. Blood samples were centrifuged for 15 minutes at 3000 rpm to obtain blood serum. The examination of blood levels of serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), lactate dehydrogenase (LDH), tryglicerides (TG), and total cholesterol (T-Chol) using clinical chemistry automatedanalyzer. Assessment of liver biomarker resultwas assisted by a clinical pathologist.

Liver Anatomy and Histopathology Examination Procedure

To assess liver rat changes, these organs were removed, cleaned from the surrounding tissue and washed with PBS (pH 7.4) to remove the remaining blood. Liver were weighed and observed. Documentation using a Canon EOS 80D DSLR. After that, these organs was put in a pot and fixed with 10% NBF with a ratio of 1:10, then sent to the Anatomical Pathology Laboratory, Airlangga University to make histopathological preparations with Hematoxyline and Eosin staining. Observation of the preparation with an Olympus CX23 binocular microscope assisted by an anatomical pathologist. For each preparation, number of normal, degenerative and necrotic cells were counted infive visual fields. In addition, portal, periportal, and lobular inflammatory cells and Kupffer cells were also observed.

Statistical Analysis

The research data areprocessedindependently using SPSS for Windows version 18.0 at least three times. The results are expressed as the mean ± standard deviation and display the anatomy and histopathological image.Independent t-test was used to show the difference between the control (C) and experimental groups (E). P value <0.05 was considered significant.

Ethics Approval of Research

Received ethical clearance from the Health Research Ethics Committee of Widya Mandala in Surabaya, with a reference number

136/WM12/KEPK/DOSEN/T/2021.

Results

Effect of polyvinyl chloride microplasticson biomarker of liver function in blood

Average level of blood SGOT (E=136±29,3; C=128±19,3), SGPT (E=76±11,1; C=67±7,7) and TG (E=91±40,8; C=65±21,2) in white rat blood given microplastics appeared to be slightly higher than the control group. Meanwhile, the levels of LDH (E=746±371,5; C=790±236,1) and T-Chol (E=45±5; C=52±12,6) were lower than the control group. These results were visually summarized in Figure 2. However, the results of the independent sample t-test analysis showed that there was no difference between groups.Significant values on SGOT (p =.586), SGPT (p =.103), LDH (p =.794), TG (p =.167) and T-Chol (p =.189) at the 95% confidence interval showed p value >0,05.

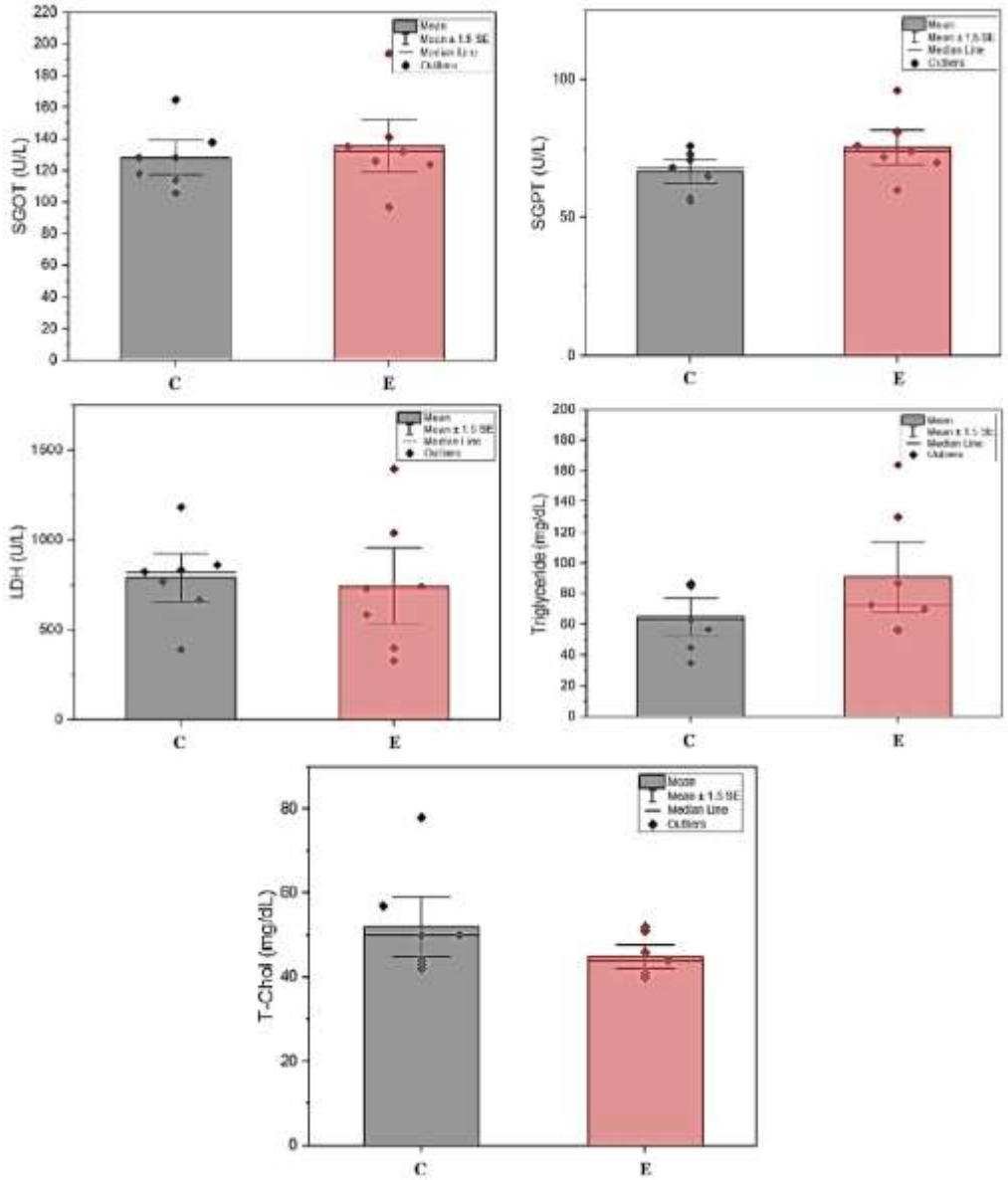

Figure 2. Differences in levels of SGOT, SGPT, LDH, TG, and T-Chol in the blood of white rats in control (C) and experimental (E) group

Effects of polyvinyl chloride microplasticson gross anatomy of liver

On gross anatomy observation of the liver, it was known that both groups had bright red liver surface color, smooth touch, no nodules, sharp edges of the liver, no visible hematoma, soft consistency, and eight lobes (Figure 3). The mean± standard deviation liver weight for experimental group was 9.6±1,6grams, while control group was 8.7±1,4grams. Based on independent t-test, there was no significant difference between the both groups (p =.294).

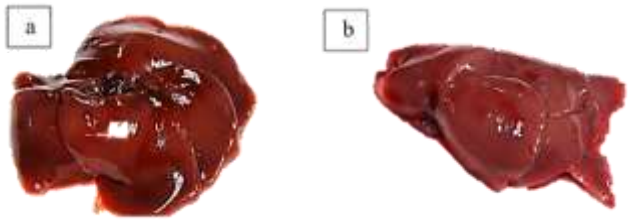

Figure 3. Gross anatomy of the liver of white rats, a) Control group; b) Experimental group

Effects of polyvinyl chloride microplasticson hepatocellular damage

On figure 4, hepatocellular changes were also more severe in experimental group, range between hepatocytes degeneration (C=119,5±34,6; E=186,7±11,5) to cell death (C=13,7±5,1; E=38,5±12,7), while normal hepatocytes were markedly noted in the control group (C=126,6±31,5; E=17,5±9,5). Statistical analysis showed that there were significant differences in the number of normal hepatocytes (p =.001), the number of degenerated hepatocytes (p =.002), and the number of cells undergoing necrosis (p =.001) between groups.

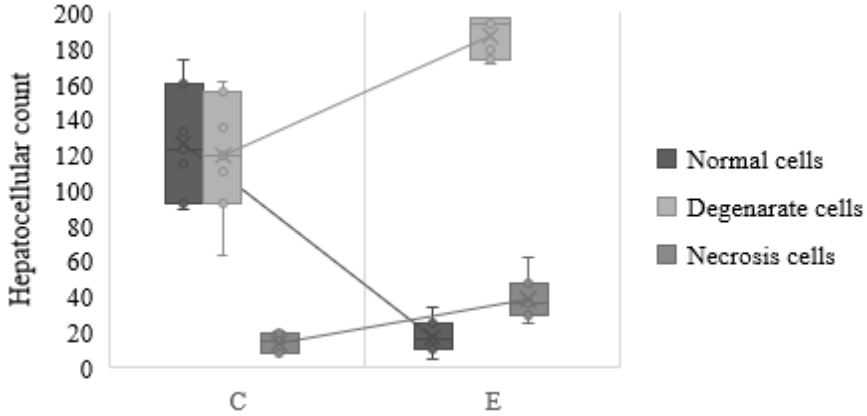

Figure 4. Differences in the number of hepatocellular in control (C) and experimental (E) group

Figure 5 shows various histological alterations, especially in the experimental group. Mild to moderate portal and periportal inflammation was observed in both groups (Figure 5a and 5b) at 100x magnification. However, small clusters of mononuclear cells infiltration indicating the lobular inflammation were more commonly found in the experimental

group.At 400x magnification of microscope, Figure 5c as histopathological image of the control group showedconsisted mostly of normal hepatocytes, while Figure 5d as histopathological image of the experimental groupshowed prominent hepatocellular changes.In Figure 5e, lobular inflammation (white arrow), necrosis (dark arrows) and vacuoles in cytoplasm indicating hepatocytes degeneration (blue arrows) are shown. Figure 5f shows apoptotic cell (white arrow), necrosis (dark arrows) and some scattered Kupffer cells (blue arrows).

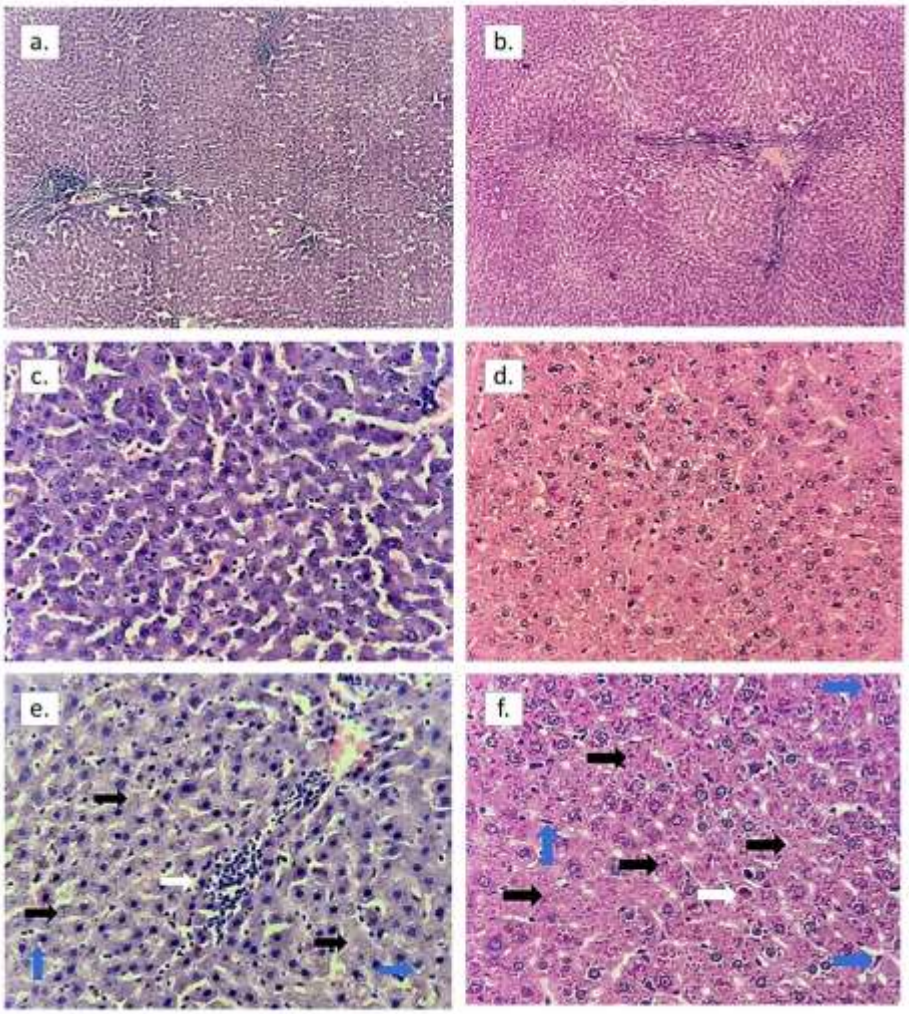

Figure 5. Representative histopathological images of white rat liver tissue sections, stained with Hematoxyline Eosin.

Description: 5a) Control group, 100x magnification; 5b) Experimental group, 100x magnification; 5c) Control group, 400x magnification; 5d) Experimental group, 400x magnification; 5e) Control group, 400x magnification, lobular inflammation (white arrow), necrosis (dark arrows) and vacuoles in cytoplasm indicating hepatocytes degeneration (blue arrows); 5f) Experimental group, 400x magnification, apoptotic cell (white arrow), necrosis (dark arrows) and Kupffer cells (blue arrows).

Discussion

In this study, the occurrence of hepatocellular degeneration was prominent (Figure 4). Hepatocellular degeneration occurs in response to environmental changes. Ingested microplastic particles are translocated through the gastrointestinal tract and will experience persistence, bioaccumulation and are toxic to the liver, which might play a critical role in the initiation of this physiological process (Deng *et al.*, 2017; Wright and Kelly, 2017). The impact of microplastics can be caused by additional chemicals during the production process or absorbed in nature such as phthalates (Sun *et al.*, 2016), bisphenol-A, polychlorinated biphenyls, polybrominated diphenyl ethers, polycyclic aromatic hydrocarbons, dichloro-diphenyl-trichlorethane, nickel, and lead (Wright and Kelly, 2017; Widianarko and Hantoro, 2018). In addition, microplastics also induce oxidative stress due to free radicals generated by plastic making materials, namely carbon polymers with oxygen, nitrogen, chlorine and sulfur, causing metabolic disorders in the liver, especially energy and fat metabolism (Chang *et al.*, 2020).

Microplastics intake into the animal's body does not directly cause a negative impact (Wright and Kelly, 2017). The toxicity of microplastics is highly dependent on the size and concentration of particle plastics that enter the body of living things (Wright and Kelly, 2017; Hwang *et al.*, 2019; Wu *et al.*, 2019; Campanale *et al.*, 2020).Microplastic particles diameter of <20µm is a potential health hazard. Inconsistent results regarding concentration (dose) are still controversial in several studies (Hwang *et al.*, 2019; Wu *et al.*, 2019).This study used microplastic particles with a diameter of <20 µm with a dose of 0.5mg of microplastic particles per day, showing the presence of cell death in the liver white rats but not showing significant differences in liver function levels or gross changes in liver anatomy between the control and experimental group.

Liver is the first line organ detoxification in the body of living things. Damage to the liver causes microplasticstoxicity to attack other organs in the body such as cardiac (Li *et al.*, 2020), skeletal muscle (Shengchen *et al.*, 2021), neuron system (Lei *et al.*, 2018), kidney (Deng *et al.*, 2017; Wang *et al.*, 2021), intestinal system (Deng *et al.*, 2017; Liang *et al.*, 2021), and lungs (Chan *et al.*, 2020). Several other studies used microplastics smaller diameter, namely 1µm and 5µm. The microplastic particles diameter of 10µm is able to penetrate into the cell membranes of all organs, including the placenta and the blood brain barrier and then trigger cell damage reactions due to oxidative stress (Campanale *et al.*, 2020).Oxidative stress occurs when antioxidant levels in liver cells are lower than oxidant levels. Antioxidants such as superoxide dismutase, catalase and glutathione peroxidase have been shown to decrease in studies with microplastics (Deng *et al.*, 2017; Shengchen *et al.*, 2021).

Exposure to oral microplastics with a size of 0.5µm and 50µm as much as 1000µm/L in rats for 5 weeks reduced triglycerides and total cholesterol levels. This is due to a decrease in the expression of several key genes that play a role in the process of lipogenesis and triglyceride synthesis in the liver (Lu *et al.*, 2018). Disorders of energy and fat metabolism in mice exposed to microplastics were characterized by a decrease in adenosis triphospateand an increase in lactate dehydrogenase which was then confirmed by a decrease in the relative weight of the liver (Deng *et al.*, 2017). There is a discrepancy between the results of the study and this current study. In this current study, there has been a change in the profile of liver function where the white rats group given microplastics decreased lactate dehydrogenase (LDH) and total

cholesterol(T-Chol) levels, while serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), and triglycerides (TG) tended to be higher (Figure 2), but in the results of the different tests did not show a significant difference. Likewise with gross anatomy, there was an increase in liver weight in the group of white rats which was given microplastics. This could be due to differences in the type, size, and dose of microplastics given to the group E, even though exposure was carried out for the same duration. In this study, it was found that hepatocellular degeneration was more prominent than necrosis. This causes the difference in biomarkers of liver function and liver weight in the two groups is still not statistically significant. Degeneration is a minor injury to hepatocyte cells due to microplastic particles. This injury only reversibly disrupts cellular metabolic processes, but if exposure to microplastics continues, cell death will occur. Therefore, to obtain significant results, a longer exposure time is required.

There are two mechanisms of hepatocellular damage due to microplastics, namely oxygen dependent and oxygen independent mechanisms. Oxidative mechanisms will produce toxic oxygen metabolites as free radicals, while non-oxidative mechanism is played by the inflammatory process. The presence of this mechanism in the liver white rats due to microplastics intake is an appropriate pathological mechanism to explain how hepatocyte cells degenerate and die (Jaeschke, 2011). Oxidative stress will trigger deoxyribonucleic acid damage through the apoptotic pathway, and oxidize lipids in cell membranes through the necrosis pathway (Zhang *et al.*, 2016). Inflammation will increases intracellular lysosomes activity resulting in excess of lysosomes which is in line with the length of exposure time (Avio *et al.*, 2015). This excess lysosome results in damage to the membrane of the lysosomal system and leads to lysosome is released into the cytosol. Lysozyme will digest the hepatocyte cell membrane system and intracellular organelles and eventually cause hepatocyte necrosis (Škop *et al.*, 2012; Ueno and Komatsu, 2017). However, before hepatocytes die, it is suspected that hepatocytes experience oncosis, namely the process of hepatocyte hypoxia that interferes with the formation of adenosis triphospateand impaired intracellular ion transport in the liver. The disruption of the sodium pump causes an osmotic difference which allow water enter to the intracellular space. Excessive water (swelling) causes hepatocyte cells to rupture and die (Quesnelle*et al*., 2015). This explains how the mean liver weight of group E is higher. In this study, we assumed that hepatocellular necrosis and apoptosis occur simultaneously, which is called necroptosis. However, it needs to be further proven by examining necrostatin(NEC) biomarkers such as NEC-1 and NEC-3.

This study used only one dose of polyvinyl chloride microplastic, so the analysis of the results may be biased. Another limitation, the presence of microplastics in the blood as a biomarker of exposure was not measured. For future research using microplastics in animal study should use more varying groups, doses, and a bit longer treatment time. The effective dose of microplastics causing oxidative stress in white rat cells is 5 mg particles/L (about 7.18×1010 particles/L). Rats consume 20% water daily based on their body weight. Equivalent to 0.15mg of dry powder microplastic particles per day in rats weighing 150grams.Dose 2mg particles/L is an estimate of the number of microplastic particles in the aquatic environment.

Conclusion

Oral intake of polyvinyl chloridemicroplastics with <20 µm in size and a dose of 0,5mg per day for up to 28 days in white rats caused hepatocellular injury. Histopathological picture shows prominent hepatocyte degeneration rather than necrosis. Lobular inflammation is clearly seen, and more Kupffer cells are found.The experimental group's liver weight tended to be heavier compared to the control group, although not statistically significant. On examination of blood liver function biomarkers also did not show statistical differences between groups.

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