In vivo study of 8-OHdG as a biomarker DNA damage by combining the exposure of nonyl phenol and copper using ELISA technique

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Abstract. Nonylphenol (NP) is an environmental contaminants produced by alkylphenol ethoxylates which is non-ionic surfactant. NP has recently been found in foods derived from antioxidant hydrolysis of tris nonylphenyl phosphite which is used as a heat stabilizer in the manufacture of polymer food packaging materials, such as styrene, vinyl polymer, rubber and polyolefins. Copper (Cu) is a redox metal which in the normal dose is an essential mineral that needed by the body, but excess Cu will cause damage in some organs. Both NP and Cu can produce OH radicals in the body, which means that OH radicals will potentially attack DNA and thus form a biomarker of DNA damage such as 8-OHdG. This study of DNA adducts of 8-OHdG formation due to NP as a xenobiotic and Cu as metals. In this research, the eight-month-old Sprague Dawly rats weighing 200–300 g were randomly divided into three groups (Control, Cu, NP, and NP + Cu, n = 5). The tested rats were orally treated every 24 h for 28 days. Finally, the collected plasma was tested using ELISA technique to study the 8-OHdG content. Based on the results, it was shown that both NP and Cu metals could trigger the formation of 8-OHdG. The highest concentration of 8-OHdG formed was the exposure group of NP + Cu in day 14 with a concentration of 1.3412 ng/mL.

Keywords: Nonylphenol, oxidative stress, 8-OHdG, copper, ELISA

1. Introduction
In the past century, the fast growing industries and human population have led the researchers to develop various types of chemical synthesis to be applied in the foods and beverages industries, which causes pollution to be likely emerged and harming the aquatic systems, leading to the environmental damage that have impact on the aquatic biota and humans health [1]. One of the most hazardous pollutants is endocrine disrupting chemical (EDC). This substance is dangerous because it has a structural similarity to endocrine hormones. Chemicals that are included in the endocrine disruptors category are varied, namely polycyclic aromatic hydrocarbon group, pesticides, metals, solvents, plasticizers, and detergent. These substances can promote an oxidative stress which characterized by the formation of excess radicals caused by the inhibition of antioxidant enzyme activity to cell damage due to changes in metabolism of endocrine disrupting substances. In addition, high concentrations of EDC and continuous exposure will increase the risk of cancer [2].
NP is considered as a strong xenoestrogen in the aquatic environments because of its low solubility (4.5 mg/L at 25 °C) so that NP is not readily degraded in the environment and often used in the manufacture of Nonyl Phenol Ethoxylates (NPEs) [3]. NPE is a non-ionic surfactant found in plastics, solvent, detergent, paint and plasticizer. NP exposure to organisms can disrupt the endocrine system, which results in the hormonal and reproductive organ development to become imbalance [4]. Chronic exposure of NP can increase the state of oxidative stress by increasing the reactive radicals such as hydroxyl radicals •OH. This reactive species can interact with biomolecules, namely DNA which causes DNA damage which, if not repaired, can lead to carcinogenesis [5].

Oxidative stress describes a higher level of radicals than antioxidants with the ability of biological systems to detoxify the reactive intermediates or repair the damage caused. Radicals are produced naturally in the body during oxygen metabolism and has an essential role in cell signaling and homeostasis. Metal ions produce intracellular radicals directly or indirectly, where the Fenton reaction is one of the best-known mechanisms. During this reaction, the transition metal ion reacts with H2O2 to produce toxic •OH and oxidize the metal ion [6].

Several metals can produce free radicals, for example, Cu metal. This metal is a cofactor of many enzymes such as cytochrome c oxidase, ascorbate oxidase or SOD, which are involved in the cellular redox mechanisms [7]. Cu can cause an oxidative stress through two mechanisms: first, Cu can accelerate the formation of ROS through the Fenton or Haber-Weiss reaction [8]; second, significant increase of Cu exposure will reduce GSH levels [9]. Several guanine oxidation products at C-8 position have been used as markers of DNA damage. 8-OHdG is a biomarker of DNA damage caused by oxidative stress both in the urine and plasma due to the ease in which the guanine bases interact with hydroxyl radicals.

This study was conducted to analyze the mechanism of the formation of 8-OHdG due to NP and Cu exposure to Rattus norvegicus with the autoxidation process approach through the reaction of Fenton with a simple ELISA technique.

2. Materials and method

The research was carried out using a scientific approach to analyze the phenomenon of the increase of cancer risk in the presence of biomarkers of DNA damage in-vivo using the ELISA kit method on the test animals plasma exposed to nonylphenol as xenobiotics and Cu metals as catalysts for the formation of ROS. This study was approved by the ethics committee at our institute.

2.1. Test animal preparation

The test animals were kept in cages and consisted of 5 mice in each cage representing each treatment group. Each treatment was given an identification tag so that it was easy to distinguish between treatment groups. Test animals were acclimatized for about two weeks to be able to adapt to the new cage environment. During the acclimation process, the test animals were given pellet feed and boiled water for drink. Animal cages were cleaned three times a week with disinfectant and replaced the base of the cage. The lighting of the room where was conditioned with 12 h light and 12 h dark at room temperature around 27–28 °C.

2.2. 8-OHdG formation in the control group, Cu, NP and NP + Cu

The sunflower oil, Cu solution 4 mg/kg bw, NP solution 25 mg/kg bw, and a mixture of Cu and NP solution were prepared for the oral feeding process. Rats were grouped into 4 groups (control, Cu, NP, NP + Cu) then given feed in the form of pellets, drinking ad libitum and every day each group was treated differently from one another orally as much as 10 mL/Kg bw for 28 days.

2.3. Blood sample collection

Before drawing blood from rats, the test animals were anesthetized using 0.1 mL of ketamine and xylazine for 2-4 min. The rats were weighed and then blood was drawn through the sinus orbital eye
using a hematocrit pipette of 1–1.5 mL and put in a sterile tube containing EDTA. The blood was centrifuged at a speed of 3500 rpm for 20 min at a temperature of 20 °C. Plasma was separated and stored in a freezer at -20 °C until the measurement process.

2.4. Analysis of 8-OHdG in plasma
Analysis of the 8-OHdG formation in plasma samples from rat test animals was performed using the ELISA kit method from CUSABIO. First, all reagents, standards, and samples were prepared. Then, 100 μL standard and sample were added to the well plate and covered using para-film. Then, incubated for 2 h at 37 °C. Next, the liquid in each well plate was removed and added with 100 μL biotin-antibody to each well plate, then covered with para-film and incubated for 1 h at 37 °C. After incubation was complete, the liquid in the well plate was removed and washed using a wash buffer and let stood for 2 min. Washing was done for 3 times. Then, 100 μL HRP-avidin was added to each well plate and covered with para-film. Then, incubated for 1 h at 37 °C. After completion, the well plate was washed again using wash buffer 5 times. Then, 90 μL TMB substrate was added to each well plate and incubated again for 15 min at 37 °C. After incubation was complete, a stop solution was added to each well plate. Then, the optical density was determined on each well plate for 5 min using spectrophotometer at a wavelength of 450 nm.

3. Results and discussion
The in vivo study began with an acclimation process that lasted for 3 weeks. The Health Research Ethics Committee of the Faculty of Medicine, Universitas Indonesia has approved the in vivo study method to be carried out, with an ethical approval number: 1112 / UN2.F1 / ETIK / 2018.

3.1. Observation on the test animals
The exposure on rats used in this study is sub-chronic exposure, which is the exposure in low concentrations for a short period of time. Exposure of NP is 25 mg/kg bw above its NOAEL value, while Cu metal exposure is 4 mg/kg bw. Changes of rats’ body weight, before and after exposure can be seen in figure 1. Based on the observations, the body weight of rats given xenobiotic exposure tends to decrease. The weight of the control group rats increased from 214.2 g to 251.8 g at the end of the exposure. However, in the Cu exposure group, the body weight of rats decreased from 229.5 g to 190.95 g. This shows that sub-chronic exposure of Cu affects the body weight of rats. The body weight of rats in the NP exposure group also decreased from 260 g to 238 g. The same thing happened in the combination of NP and Cu where the weight loss from 266 g to 236.2 g was experienced, so it was seen that the sub-chronic exposure of both NP and Cu can cause the weight lost. Previous studies of organ damage test using NP for 8 weeks showed micronuclei induction in erythrocyte cells. In that research, erythrocytes in the blood of females experienced a resistance at a dose of 25 mg/kg bw [10]. The accumulated NP will cause a decrease in sperm quality due to the nature of NP which resembles the hormone estrogen and will result in the decrease total number of sperm cells produced thereby affecting the weight of the testicles of test animals [11].

3.2. Analysis of plasma in vivo studies
The formation of 8-OHdG in the in vivo study was analyzed using a sandwich type ELISA kit derived from CUSABIO. Before analyzing using an ELISA kit, blood taken from rats was centrifuged at 20 °C at a speed of 3500 rpm for 15 min until blood plasma was obtained. Then, the plasma was stored in a freezer at -20 °C until the analysis of the 8-OHdG compound using an ELISA kit was carried out. A sandwich type ELISA was used to identify the specific antigens, 8-OHdG in the sample. A microplot surface has been prepared with bound 8-OHdG antibodies that are known to be able to capture the desired antigen. Primary antibodies were added 8-OHdG to the samples such as ‘sandwich’, then biotin antibodies were added to bind with the HRP-avidin enzyme (figure 2). Unbonded enzyme antibody conjugates were cleaned using a buffer. TMB substrates were added and
converted enzymatically to produce quantifiable colors. One of the advantages of using specific antibodies is that they can eliminate steps to purify antigens from other antigen mixtures, making it easier to test and increase their specificity and sensitivity [12].

The 8-OHdG compound, which is a product of DNA cutting by DNA repair, will be distributed through the blood and excreted in the urine without further metabolic processes. Analysis of 8-OHdG compounds in blood plasma as a biomarker of DNA damage in the body is carried out because of the ease of sampling and handling.

3.3. 8-OHdG formation in the control group

The control group in this study was a group of rats that were only snapped using sunflower oil without the addition of any compound. Although not exposed to xenobiotics, rat can experience oxidative stress due to other factors such as the environment and the state of rat during the study. The control group also produced 8-OHdG and can be seen in figure 3.

Naturally, cells can experience oxidative stress because they can produce ROS. Each cell in the human body can process 1021 oxygen molecules each day with 1% metabolism producing ROS. Based on the excretion of 8-OHdG in urine, it can be estimated that about 20,000 nucleosides in DNA are damaged in each human cell per day [13]. Therefore, even though mice are not rounded with xenobiotics, it will still produce 8-OHdG, but tend to decrease due to metabolic processes in the body working to repair 8-OHdG products that are formed.

![Figure 1. Body weight of rat before and after exposure.](image1.png)

![Figure 2. 8-OHdG standard calibration curve](image2.png)
3.4. 8-OHdG formation in the NP group
Exposure to NP in rats will produce an 8-OHdG DNA adducts as shown in figure 4. Based on the graph, NP exposure tends to decrease after the first week of exposure. This is partly due to the blood lysis that occurs during the centrifugation process, because in that week the centrifugation process is carried out at room temperature. However, it shows an increase of 8-OHdG products in week 2. In weeks 3 to 5, the 8-OHdG products produced tend to be consistent, it shows that the presence of xenobiotics that enter the body will continuously produce an excess ROS so that enzymes that act as antioxidant are no longer able to neutralize the radical products so that 8-OHdG will continue to form.

Exposure to NP has been shown to cause oxidative damage to DNA [14]. NP induces mitochondrial damage, which will lead to endocrine damage [15]. NP itself can undergo an autoxidation process by producing radicals that can trigger oxidative damage to DNA. However, as is well known, NP is rapidly hydrolyzed by the esterase enzyme.

3.5. Effects of Cu exposure on the formation of 8-OHdG
Exposure of Cu in rats will produce an 8-OHdG DNA adduct, as shown in figure 5. Based on the figure, exposure of Cu decreases after the third week during the experiment. This is partly because of the blood lysis that occurs during the centrifugation process, which can interfere with the resulting 8-OHdG concentration.

Like other studies, DNA adduct in the puppy’s liver are formed due to the increase of oxygen exposure accompanied by both Fe and Cu content found in food consumed by the mother [16].
3.6. The exposure effect of the combination of NP and Cu metals on 8-OHdG formation

The effect of NP and Cu metals on the formation of 8-OHdG is shown in figure 6. The exposure combination of NP and Cu results in the formation of 8-OHdG. As explained in the previous discussion, both NP and Cu can cause oxidative damage to DNA. The concentration of 8-OHdG in NP with Cu metals also decreased in the third week during the experiment because of blood lysis during the centrifugation process. This exposure group experienced the most significant increase in 8-OHdG and showed the most significant weight loss. That is because the synergy correlation between xenobiotics and metals on the production of 8-OHdG.

3.7. Comparison of the Formation of 8-OHdG in each group

Comparison of the formation of 8-OHdG in various exposure groups at 28 weeks can be seen in figure 7. Based on the pictures, the control group had the lowest 8-OHdG concentration compared to other exposure groups. This shows that both NP and Cu metal play a role in the formation of 8-OHdG.

Based on figure 7, exposure from a combination of NP and Cu causes the formation of 8-OHdG with the highest concentration compared to other groups. This is because the correlation between xenobiotics and metal is highly involved in redox reactions in the body and the excess of this metal will facilitate the formation of radicals in cells such as hydroxy radicals.

NP will inhibit the activity of SOD enzymes so that radicals formed from oxygen cannot be neutralized again by antioxidant enzymes. These radicals will interact with DNA bases and form 8-OHdG, as well as Cu. Excess Cu will allow hydrogen peroxide to interact with Cu (I), thus forming...
a hydroxyl radical. That is why a combination of NP and Cu exposure will increase the yield of 8-OHdG formation than other exposure groups.

Figure 8 shows the formation of radicals in the body. Oxygen will be reduced to superoxide radicals in the presence of the enzyme cytochrome P450. Superoxide radicals will be neutralized by the SOD enzyme into H2O2 products. H2O2 neutralization process with the enzyme catalase will produce H2O and O2 can take place. However, H2O2 also has the potential to react with Cu to produce OH radicals. Figure 9 shows the OH radicals that are formed to be likely interact with DNA. Guanine is one of the bases mostly attacked by OH radicals because it has the highest oxidation level compared to thymine, cytosine, and adenine. Guanine position C number 8 is the preferred position for the OH radicals because of the open planar position steric effect compared to C positions 4 and 5. So, 8-OHdG is the most effective biomarker for identifying the DNA damage.

![Figure 7. Comparison of the formation of 8-OHdG in various exposure groups.](image)

![Figure 8. Radical formation scheme in the body's metabolism.](image)
Figure 9. 8-OHdG formation scheme from radical hydroxyl exposure.

4. Conclusion
There was an increase in the levels of DNA adducts (8-OHdG) in plasma during the time of exposure in the NP exposure group and the combination of NP and Cu metal exposure group. The synergistic effect between the Fenton-like reaction with NP in the formation of DNA 8-OHdG is shown by the increase level of 8-OHdG in the plasma of the NP and metal combination groups compared to the both NP and Cu alone exposure groups.

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