Chapter

In Vivo Study of DNA Adduct (8-OHdG) Formation of *Rattus novergicus* Using Bisphenol a through Fenton-Like Reaction and Nickel (II) as Cancer Risk Biomarker

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Abstract

Bisphenol A (BPA) has been used in many consumer products including plastics, and food packaging. There is the evidence that Bisphenol A have potential to cause oxidative stress by disturbing the redox status in cells. We have conducted the in vivo study of BPA and Ni(II) exposure to *Rattus novergicus* and confirmed the formation of DNA adduct 8-OHdG as biomarker of oxidative stress and cancer risk. Subacute dose of BPA (2 mg/kg BW) and Ni (II) metals (0.1 μg/kg BW) have been exposed to animal test for 28 days. We collected the urine sample of animal samples every week. The formation of 8-OHdG found in urine of animal samples monitored by Liquid Chromatography–Mass Spectrometry (LC–MS/MS). The result of this study indicates that levels of 8-OHdG in animal samples exposed to BPA and BPA-Ni (II) increase every week. However, levels of 8-OHdG in animal samples exposed by BPA-Ni (II) is less than levels of 8-OHdG in animal samples exposed by BPA only. This can be happened because Ni (II) given to animal samples are not in the excessed levels, therefore the synergic effect of BPA and Ni (II) has not already been seen. The hydroxyl radical can cause oxidative DNA damage and interact with DNA guanine base by producing DNA adduct 8-hydroxy-2′-deoxyguanosine (8-OHdG). This book aimed to obtain information regarding in vivo study of BPA and metal ions exposure can generate hydroxyl radical as a dominant form of Reactive Oxygen Species (ROS) that can interact with macromolecules such as DNA and form DNA adduct as biomarker of oxidative stress and cancer risk.

Keywords: BPA, Nickel (II), 8-OHdG, in vivo study

1. Introduction

Bisphenol A (BPA) is a common chemical compound with the highest production volume in worldwide [1]. BPA is a synthetic chemical used as a monomer for the manufacture of polycarbonate plastic, and also as an intermediate in the

synthesis of epoxy resins. Humans have considerable possibilities for direct exposure to BPA from plastic beverage containers and from saliva patients receiving dental fillings [2]. Humans are exposed to BPA through their diet, inhalation of household dust, and dermal exposure [3]. Bisphenol A (BPA) is a known endocrine disruptor [4]. It have a potent reproductive and genotoxic agent and affects the normal physiological functions [5]. Oxidative stress has been proven as a basic mechanism of BPA toxicity in animal models for years. Bisphenol A have the potential to cause oxidative stress by disturbing the redox status in cells [6].

On the other side, heavy metals Nickel (II), may contribute to the formation of ROS in humans. Humans may be exposed to nickel if involved in nickel production or through contact with everyday items such as nickel-containing jewelry, tableware, and cigarettes. Ni (II) is toxic and carcinogenic in animals and humans [7] and has been classified by the International Agency for Research on Cancer (IARC) as a carcinogen in humans [8]. This can be happened because nickel ions (II) also have the ability to form hydroxyl radicals through Fenton-like reactions [9].

The hydroxyl radicals formed can cause oxidative stress. Such increased oxidative stress can lead to DNA damage which can result in carcinogenesis [10–13]. In DNA repair, additional products are formed and released into the bloodstream and appears in the urine without further metabolism. The 8-hydroxy-2'deoxiguanosine (8-OHdG) compound is one of the adducts derived from the hydroxyl radical attack

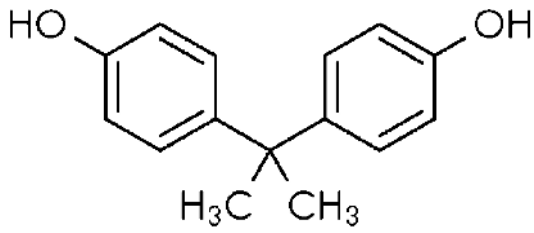

Figure 1.
Structure of bisphenol A.

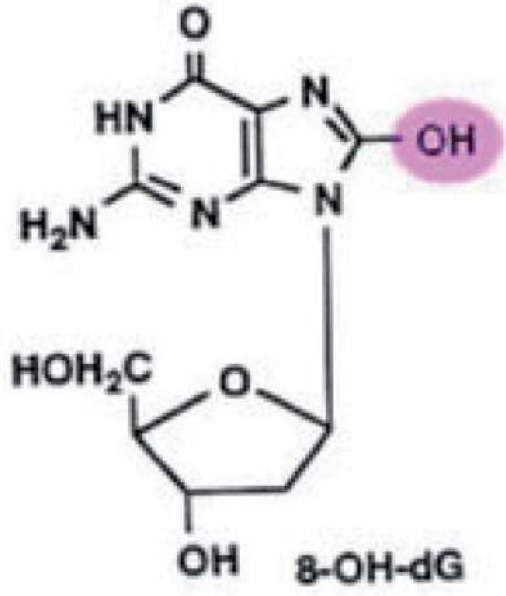

Figure 2.
Structure of 8-OHdG.

of deoxyguanosine residue and has been commonly used as a biomarker of oxidative damage to DNA [14]. With the detection of 8-OHdG, the risk of cancer can be detected earlier so that it can be further minimized.

This study aims to determine the formation of 8-OHdG which is an indicator of DNA damage due to exposure from BPA and Nickel (II) metal (**Figures 1** and **2**). The 8-OHdG in vivo formation was analyzed in the urine of Sprague–Dawley rats. For analysis of 8-OHdG, pre-treatment method of urine sample with SPE (Solid Phase Extraction) was performed. Furthermore, an analysis using LC–MS/MS instrument is used to view and analyze the formation of 8-OHdG as a proof of exposure of those toxic substances which can lead to DNA damage.

2. Experimental methods

2.1 Materials

The materials used for the rats' exposure rats are Bisphenol A (BPA), Nickel (II) metal in the form of $NiCl_2.6H_2O$. 8-hydroxy-2′-deoxyguanosine, ammonium acetate, acetic acid, acetonitrile, aquabidest, methanol, and SPE cartridge SPE *SUPELCO-Discovery® DSC*-18.

2.2 In vivo study

2.2.1 Experimental animals

In this study, the experimental animals used were 8-weeks old male white rats from the Sprague–Dawley strain, weighed approximately between 120 and 220 grams obtained from the Faculty of Animal Husbandry of Non Ruminants and Animal Hope, Bogor Agricultural Institute, Bogor. Prior to the research, rats were acclimatized for 7 days so that they could adapt to the new environment. Then, all of the rats were adjusted to the optimal weight for 14 days. The food were pellets and placed in a cage while drinking water were given at the drinking place by pipette - *ad libitum*.

2.2.2 Xenobiotics exposure to experimental animals

Rats as experimental animals were divided into several groups. The groups were: Group A (group of rats with no exposure or control), group B (group of rats exposed to BPA), and group C (group of rats exposed to BPA and Ni (II). Each group consists of 5 rats. The study was conducted for 28 days. All the exposures were done by oral route (syringes equipped with a blunt-shaped or blunt-shaped cannula). The dose for each group of experimental animals are given in **Table 1**.

2.2.3 Urine sampling

The urine samples were used for the 8-OHdG analysis. For urine collection, rats were placed in a metabolic cage. When the urine is fully contained in the container located in the metabolism cage, urine is transferred to the micro-tube and then stored at −20°C freezer until the time for measurement using LC–MS/MS.

2.3 LC–MS/MS

Samples were analyzed on a 20A high performance liquid chromatography system (Shimadzu, Japan) coupled with an AB Sciex Q-Trap 3200 mass

Dose in each group	BPA	Ni (II)
Group A	—	—
Group B	2 mg/kg bw	—
Group C	2 mg/kg bw	0,1 µg/kg bw

bw: body weight.

Table 1.
Dose of xenobiotics exposed to experimental animals.

Time (minutes)	Pump B Concentration (%)
0	0
0.5	5
1	95
2	95
2.5	5
3	0

Table 2.
Eluent gradient on LC–MS/MS.

Parameter	Precursor Ion	Q3 (Product Ion)	
	284,1	**168,2**	**140,1**
Q1	DP	43	43
	EP	4	4
MRM	CE	17	40
	CXP	3	3

Table 3.
Optimization result for LC–MS/MS instrument to measure 8-OHdG.

spectrometer and were separated on a Hypersil Gold C18 column (50 x 2,1 mm, 5 µm, Thermo Scientific). The pump used is in gradient mode. The mobile phase composition (eluent) is Ammonium Acetate 20 mM pH 4 (Pump A) and Acetonitrile (Pump B) which were adjusted automatically during elution with a gradient of 5–95% Acetonitrile solution with a certain time setting. **Table 2** shows the mobile phase (eluent) gradient during elution:

The flow rate used is 0.5 mL/min. Meanwhile, the column temperature is set at 40°C. The sample injected was as much as 10 µL and the analysis time was 5 minutes. The MS/MS parameters, including parent ion (Q1), product ion (Q3), collision energies (CE), declustering potential (DP), entrance potential (EP) and collision exit potential (CXP) were optimized. The optimized parameters are listed in **Table 3**.

Determination of 8-OHdG calibration curve was done by making standard series of each 10 µL 8-OHdG standard solution at concentration of 1 ng / mL (ppb), 5 ng / mL, 10 ng / mL, 25 ng / mL, 50 ng/mL. Measurements were made for each of these standard concentrations to obtain a calibration curve between the peak area obtained from the standard solution concentration, with a straight-line equation having

$R^2 > 0.996$. Limit of Detection (LOD) and Limit of Quantification (LOQ) limits are performed statistically by linear regression of the calibration curve. The measurement value will be equal to the value of b on the linear line y = a + bx, whereas the standard deviation of the blank is equal to the residual standard deviation (S (y/x)).

2.4 Analysis of 8-OHdG in urine samples

500 μL of urine samples were passed to the SPE SUPELCO -Discovery® DSC-18 cartridge column, previously conditioned with 5 ml of methanol and 5 ml of Ammonium Acetate. Furthermore, the cartridge was rinsed with 20 mM Ammonium Acetate pH 4: Acetonitrile (97: 3) of 5 ml. Samples are streamed to the SPE column of 500 μL. The fraction containing 8-OHdG in the cartridge is dried with N2 gas under vacuum SUPELCO VISIPREP. The fraction was added Ammonium Acetate 20 mM pH 4: Acetonitrile (80:20) of 5 ml. The elution results are then dried with nitrogen gas. A total of 10 μL samples were injected into the LC–MS/MS instrument to analyze its 8-OHdG formation.

3. Results and discussion

3.1 Determination of optimal conditions for 8-OHdG formation using LC–MS

Prior to the analysis of 8-OHdG formation in urine samples, optimization was done in advance to determine the best condition of the instrument, resulting in a performance of the method with good sensitivity. For the mobile phase, 20 mM Ammonium acetate with pH 4 and acetonitrile was used according to the method in the literature [15]. In **Figure 3**, from the standard 8-OHdG 50 ppb measurement results, it can be seen that 8-OHdG appears at retention time of 0.738 minutes.

3.1.1 Optimization of mass spectrometer condition

In LC–MS/MS instrument it takes at least two ion products with the highest intensity. This is useful for quantitative information in which the molecular weight compound is detected by the instrument in peak form, and to provide confirmation that the resulting peak actually belongs to the desired compound.

Figure 4(a) shows that the 8-OHdG compound is detected by Q1 to produce a precursor ion value of 284. Then, the ions are split in the presence of accelerated ions by the gas which in this LC–MS/MS instrument is nitrogen gas. Therefore, the

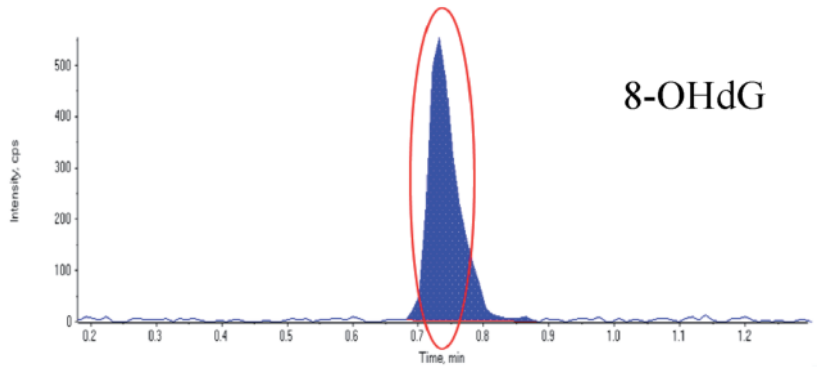

Figure 3.
Chromatogram of 8-OHdG standard (50 ppb).

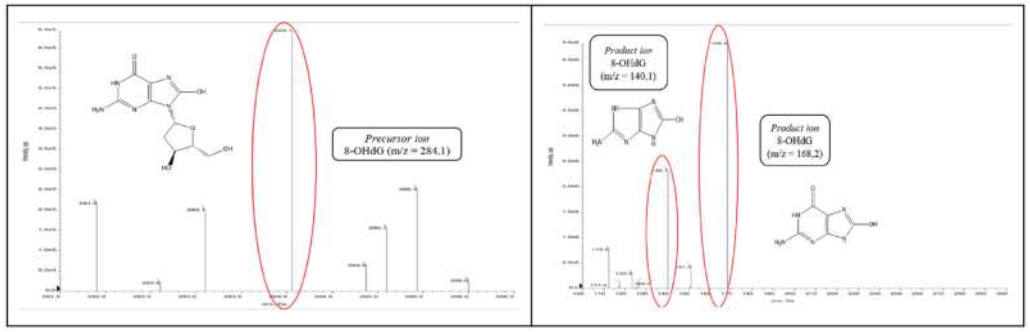

Figure 4.
(a) *The ion precursor of the 8-OHdG compound with Q1 detection (m/z = 284.1)* **(b)** *the product ion of 8-OHdG (m/z = 168.2) and (m/z = 140.1).*

precursor ions will be broken down into smaller ion fragments of molecular weight, which are further fragmented in Q3 to produce the highest intensity productions at 168.2. The transition for the selected mass spectrometer for 8-OHdG is m/z 284.1 → 168.2 for quantification and m/z 284.1 → 140.1 for qualification. Product ion 168,2 and 140,1 can be seen in **Figure 4(b)**.

8-OHdG with m/z 284,1 is fragmented into its product ions 168,2 and 140,1 according to **Figure 4(b)**. First, there is a cutting of sugar in the 8-OHdG compound, which causes 8-OHdG to be fragmented into its first product ion with m/z 168.2. Cutting or further fragmentation is on the C = O bond that causes 8-OHdG to be fragmented again into its product ion of m/z 140.1.

3.2 Verification method

The equation of straight line with R^2 = 0.9985 is obtained. Limit of Detection (LOD) is the smallest quantity of analytical concentration detectable from an analytical method while Limit of Quantification (LOQ) is the smallest accurate and meticulous quantity of the smallest calculated (quantified) concentration. The LOD and LOQ calculations were performed on the basis of a standard 8-OHdG standard calculation which had previously been performed for linearity determination, determined from the standard 8-OHdG calibration curve to the ratio of chromatogram area. From the calculation of statistics, obtained LOD value is 1,315 ppb and LOQ value is 4.384 ppb.

3.3 Analysis of 8-Hydroxy-2'-Deoxyguanosine in urine samples

3.3.1 Urine sampling and urine sample pre-treatment

This research has also gone through protocol study so that it can pass the ethical review through Medical Research Ethics Commission of Faculty of Medicine, University of Indonesia Jakarta based on letter number 0386/UN2.F1/ETIK/2018 and protocol number 18-03-0321.

Each week each rat was taken urine according to the method in point 2.2.3. Rats are placed in a metabolic cage for their urine shelter. For urine, the volume varies between 1 and 5 ml.

Before the sample is analyzed by LC–MS / MS instrument, the sample is passed first in the SPE column. SPE with column C18 allows to remove substances that interfere in the urine. The SPE method also shows considerable reproducibility and accuracy [16]. Pre-treatment of samples with SPE is necessary because if not treated first, it may interfere with measurements on LC–MS/MS instruments.

3.3.2 Effect of BPA on formation of DNA adduct 8-OHdG

After an 8-OHdG analysis was performed on the rats' urine samples of group B (rats given BPA exposure), a chromatogram was obtained with results as in **Figure 5**. 8-OHdG was detected at retention time of 0.773 minutes.

BPA exposure in rats has an effect that may lead to the formation of DNA adduct 8-OHdG. As shown in **Figure 6**, the 8-OHdG levels in rats exposed to BPA were greater than controls. Levels of 8-OHdG in rats given exposure to BPA also increased from exposure time of 1st to 4th week is from 27.041 ng/mL up to 31.220 ng/mL.

In this condition, the concentration of 8-OHdG is also above the LOD value. This is sufficient to prove that 8-OHdG as biomarker of DNA damage [17] can be formed and detected due to in vivo BPA exposure.

The results of this study indicate that BPA can increase the formation of hydroxyl radicals, which can bind to DNA to form 8-OHdG. The mechanism that causes BPA to lead to the formation of 8-OHdG is due to the fact that BPA can covalently induce the formation of ROS that can bind to DNA in vivo [18]. Metabolic conversion of BPA to DNA is catalyzed by cytochrome P450 in the liver to form 5-hydroxybisphenol [19]. Hydroxybisphenol is a catecholestrogen compound that is capable of redox cycling. Hydroxybisphenol is further oxidized to semiquinone, then the semiquinone will be further oxidized to 4,5-Bisphenol-O-quinone [18]. The catechol-o-quinone is capable of redox cycling with generation of oxidative

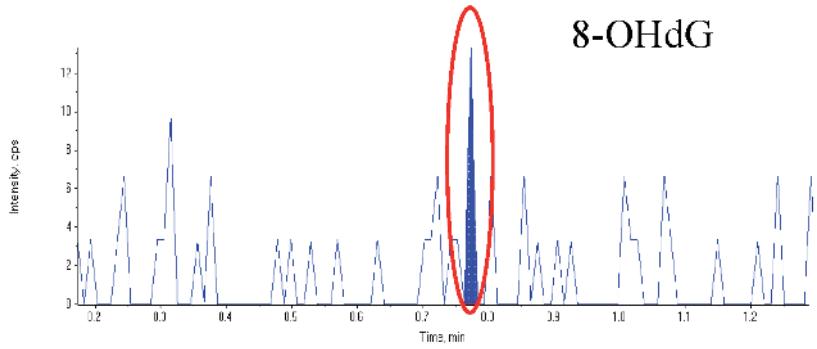

Figure 5.
Chromatogram of rats' urine samples given BPA exposure.

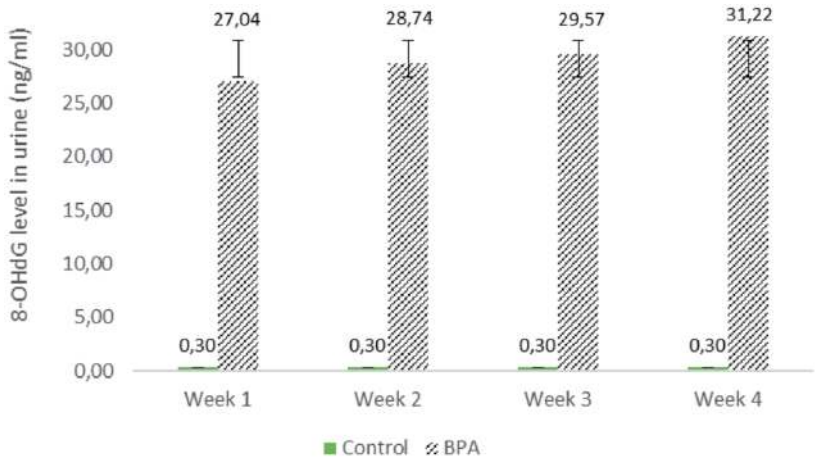

Figure 6.
The comparison graph of 8-OHdG concentrations between control rats and rats given BPA exposure.

stress (OS) and reactive oxygen species (ROS). o-Quinones are highly electron affinic with potential reduction that permit electron transfer (ET) under physiological conditions. Large quantities of ROS are sufficient to generate catalytically by small amounts of o-Quinones. There is extensive evidence for generation of ROS, which include ET by o-quinone as a plausible source. There are numerous reports on toxicity to body constituents by BPA [20]. The metabolic reaction was illustrated in **Figure 7**.

3.3.3 Effect of Ni (II) on formation of DNA adduct 8-OHdG

After an 8-OHdG analysis was performed on the rats' urine samples of group C (rats given exposure to BPA and Ni (II) metals), a chromatogram was obtained with results as in **Figure 8**. 8-OHdG in rats' urine samples exposed to BPA and Ni (II) metals were detected at retention time of 0.733 min.

Exposure to BPA and Ni (II) metals may also have an effect that may lead to the formation of an 8-OHdG DNA adduct. As shown in **Figure 9**, 8-OHdG levels in mice exposed to BPA and Ni (II) metals were greater than those of controls.

The 8-OHdG concentration in mice given exposure to BPA and Ni (II) at a dosage of 0.1 µg/kg bw also increased from exposure time of 1st to 4th week ie from 26.185 ng/mL to 28.696 ng/mL. In this condition, the concentration of 8-OHdG is also above the LOD value. This is sufficient to prove that 8-OHdG can be formed and detected due to exposure to BPA and metal Ni (II) in vivo.

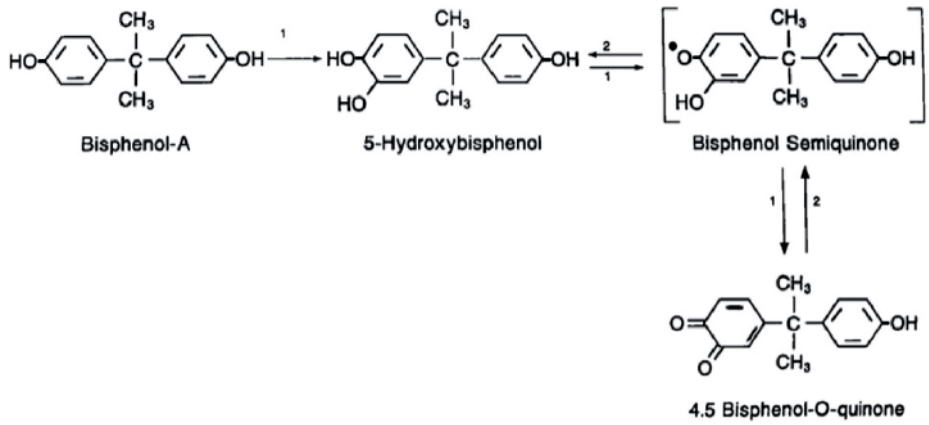

Figure 7.
Possible BPA mechanism in the human body (reprocessed). 1: Cytochrome P450; 2: Cytochrome P450 reductase. (Source: Atkinson and Roy [18]).

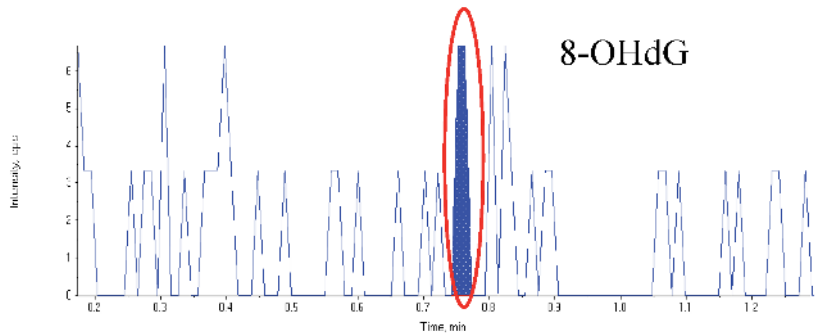

Figure 8.
Chromatogram of mouse urine samples given exposure to BPA and Ni (II).

One of the harmful effects of the nickel mechanism in the body is to induce the formation of ROS and increase lipid peroxidation in the cells [21]. Given that nickel is not an essential element for humans, it is not yet clear how nickel compounds go through the metabolic phase.

However, the resulting 8-OHdG levels of rats given exposure to BPA and Ni (II) were smaller than the 8-OHdG levels produced by rats given BPA exposure alone. The results can be seen in **Figure 10**.

This may be due to metabolic differences between rats and also because the doses given to mice are too small. A dose of 0.1 μg/kg bb resulted in an 8-OHdG increase each week in rats, but the levels remained smaller than the 8-OHdG levels obtained from rats given BPA exposure.

Oxidative stress of DNA of transition metals can possibly occurred due to the presence of free radicals produced by Fenton or Fenton-like reaction [22]. The major form of free radicals are hydroxyl radicals [17]. Nickel (II) at the excessive

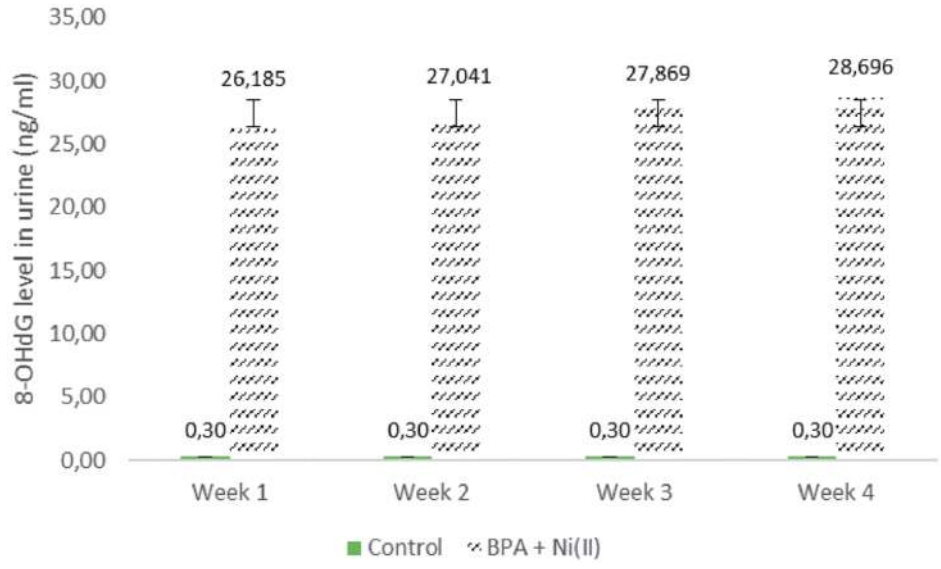

Figure 9.
The comparison graph of 8-OHdG concentrations in control and mouse rats given exposure to BPA and Ni (II).

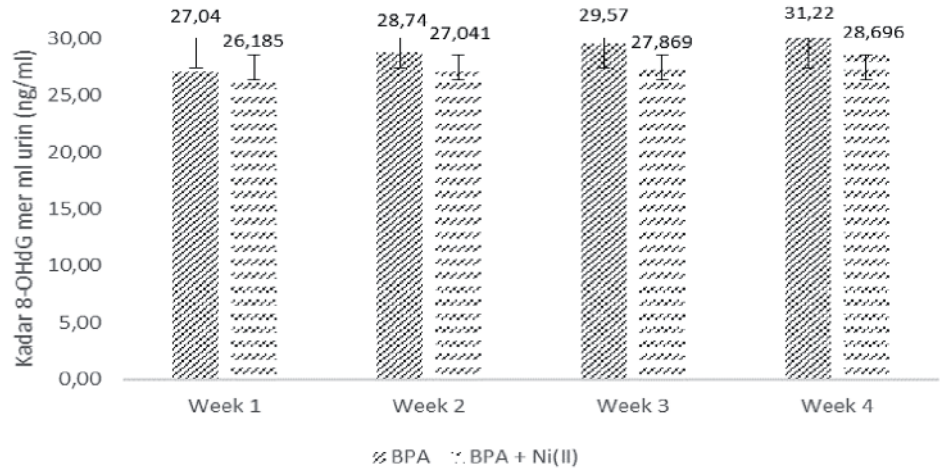

Figure 10.
Graph of comparison of 8-OHdG levels in rats given exposure to BPA and rats given exposure to BPA and Ni (II).

levels has a role in Fenton-like reaction to the formation of free radicals. Nickel (II) exposure in rats can cause a significant increase in lipid peroxidation, and a decrease of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH) [23].

Ni^{2+} is the largest nickel ionic species in the cell. There is the possibility of a Fenton-like reaction with nickel but only with the Ni^{2+} interaction with certain ligands, such as Gli-Gli-His (Glycyl-Glycyl-Histidin) or imidazole nitrogen from histidine [24]. The binding of Ni^{2+} to these ligands eliminates the oxidation potential of Ni^{2+} in which hydrogen peroxide can catalyze the oxidation of nickel to a higher oxidation state. This reaction produces a radical but the reaction depends on the binding of Ni^{2+} with certain ligands [25]. Thus, although nickel can produce oxidative stress in cells and oxidize the DNA bases, certain nickel compounds with carcinogenic potentials exhibit weak mutagenic activity in some mutation tests. However, chromosomal damage that causes mutations of a detectable gene produced by some nickel compounds may contribute to its carcinogenesis properties [26]. One interesting feature of the nickel compound is its synergistic nature with other compounds or agents that can cause cancer by its mutagenic mechanism. For example, synergic nickel with benzo [a]pirene, UV, and radiation [11]. It can also explain the absence of synergistic effects between BPA and Ni (II).

4. Conclusions

DNA adduct 8-Hydroxy-2'-Deoxyguanosine (8-OHdG) on urine sample of rats as a biomarker of DNA damage from BPA compounds and Ni (II) metals exposure, was formed and achieved the detection limit value. Levels of DNA adduct 8-OHdG in Sprague–Dawley rats after BPA/BPA and Ni (II) exposure was higher than levels of 8-OHdG in Sprague–Dawley control rats. It was also found that there was an increase in levels of DNA adduct 8-OHdG in Sprague–Dawley rats exposed to BPA and BPA-Ni (II) metals from first week exposure time until fourth week of exposure time (28 days). However, levels of 8-OHdG in rats exposed by BPA-Ni (II) is less than levels of 8-OHdG in rats exposed by BPA only. It can caused by the doses of BPA-Ni (II) metals administered to rats were in a non-excessive state, therefore the synergic effect of two toxic substances in the formation of 8-OHdG has not already been seen. There is possibility of the role of o-Quinones in metabolic cycle of BPA administered the increases of hydroxyl radical levels. And the role of Fenton-like reaction might give synergistic effect in the formation of hydroxyl radicals at the excessive level of Ni(II).

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