The Formation of 8-OHDG from Toxic Substances Trigger to Free Radicals

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ABSTRACT---- ***This study is conducted to see the profile of DNA Adduct (8-OHdG) formation as DNA damage indicators, by using calf thymus DNA incubated with toxic and carcinogenic compounds. The compounds which could trigger free radicals in this research are PAH(Benzo[a]Pyrene), and $CuCl_2$. Calf thymus DNA was incubated with Benzo[a]Pyrene and $CuCl_2$ compounds under pH and temperature variations to induce the formation of reactive oxygen species (ROS) in the process of oxidative DNA damage. From this research, all of compounds have potency to trigger the formation of DNA Adduct (8-OHdG). The ratio of absorbance to assess the purity of DNA at 260 nm and 280 nm ($\lambda 260/\lambda 280$) is measured at ~1.9. The shifted peaks at λmax are indicating changes on structures of DNA as a result of calf thymus DNA incubation with B[a]P and $CuCl_2$. The highest level of 8-OHdG results in calf thymus DNA incubation with B[a]P and $CuCl_2$ under pH 8.5 and incubation temperature at 60°C, is about 120.856 µg/L.***

Keywords--- 8-OHdG, biomarkers, DNA *Adduct*

1. INTRODUCTION

Unhealthy lifestyle and environmental conditions with air pollution can contribute to an increased risk of cancer. The potential risk of cancer continues to increase because of many sources of chemical compounds exposure that are carcinogenic. Carcinogenic chemical compound, is a compound when exposed to humans can donate free radicals in the body and if interact with biomolecules such as DNA can trigger the formation of cancer cells. Abnormal growth of cancer cells and different from normal cells due to changes in gene expression (mutagenesis) which refers to the imbalance of proliferation cell and death cell. Cancer cells can invade other tissues through the blood vessels and lymph vessels. Currently cancer is a deadly disease number two in the world, after heart disease.

Hiroshi Kasai (1986), conduct research on heated glucose which is mutagenic to Salmonella typhimurium. Furthermore, it was reported that some mutagens were detected by analyzing their adducts with guanosine derivative. When glucose is heated reacted with guanosine derivative, formation of guanosine adduct was isolated and was analyzed by high-performance liquid chromatography (HPLC). The structure of adduct was determined as the hydroxylation reaction at C-8 position of guanine residue (8-Hydroxy-IPG). The detection of adduct raises the following research on how is the mechanism of hydroxylation reaction and how is it related to mutagenesis. Subsequent research showed that various compounds which produce oxygen radicals and are known to be mutagenic and/or carcinogenic are effective in hydroxylation of deoyguanosine residue in DNA.
Carcinogenic compounds may contribute to the formation of reactive oxygen species (ROS) in the body. The most important oxygen-free radical is hydroxyl radical ($HO^{\bullet}$) which can causing damage to basic biomolecules (proteins, membrane lipids, and DNA) and lead to the generation of a variety of oxidation products. The interaction of $HO^{\bullet}$ with nucleobases of DNA leads to the generation of radical adducts. DNA repair mechanisms through mechanisms of Base Excision Repair (BER) can excrete damaged DNA. DNA is damaged and severed through the BER can be found in the form of 8-hydroxy-2'-deoxiguanosine (8-OHdG). Therefore the 8-OHdG can be used as a biomarker of carcinogenesis related to the exposure to the carcinogenic compounds.
Carcinogenic compounds such as benzo[a]Piren and compounds that can generate free radicals such as $CuCl_2$ was incubated with calf thymus DNA under variation conditions of pH and incubation temperature. This study was conducted through in vitro experience using calf thymus DNA, to prove that the compounds trigger to free radicals in individual exposure can increase oxidative DNA damage lead to carcinogenesis. The determination of 8-OHdG by the method used in this study, is expected that this study can provide information to improve cancer risk assessment in attempt to reduce or prevent the occurrence of cancer (carcinogenesis).

Therefore, this research conducted by a scientific approach and corelation studies, to analyze the phenomenon of increased risk of cancer based on adducts level using xenobiotics exposed to calf thymus DNA. DNA-Adduct (8-OHdG) is formed during in vitro reaction by incubating calf thymus DNA with B[a]P and $CuCl_2$ compounds, under pH variation (7.4 and 8.5) and incubation temperature variation (37°C and 60°C). DNA-Adduct 8-OHdG is analyzed using High Performance Liquid Cromatography (HPLC).

2. MATERIALS AND METHODS

Chemicals and reagents

Caution should be taken with benzo[a]pyrene as it is an irritant, genotoxin, mutagen and carcinogen. Calf thymus DNA 10 mg/mL (Invitrogen), 8-OHdG 500 µg/mL (Sigma-Aldrich), Benzo[a]Piren (Sigma-Aldrich), cupri klorida (Merck), TiO_2 from Sigma-Aldrich (Chemistry Department), enzim micrococcus nuclease (MN) and spleen phosphodiesterase (SPDE) enzymes (Sigma-Aldrich), methanol (Sigma-Aldrich), DMSO (Sigma-Aldrich), sodium asetate (Sigma-Aldrich), sodium succinate hexahidrate (Sigma-Aldrich), calcium chloride (Sigma-Aldrich), K_2HPO_4 (Sigma-Aldrich) and KH_2PO_4 (Sigma-Aldrich).

Instrumentation

Incubator shaker (Julobo SW22), Sonicator dan Degasser 8510 (Bronson), pH meter, ZORBAX Eclipse Plus C18 (4.6 x150 mm, 5-Micron), Membrane Filter PTFE Polypropylene backed 0,2 µm (Whatman), Eppendorf, , spectrophotometer UV-VIS 1800 (Shimadzu), and HPLC (Waters 2475)

2.1. Purity of DNA

DNA (100 µg/mL) absorbances were compared in maximum wavelength λ260 nm and λ280 nm. The purity of DNA has absorbance ratio (λ260/ λ280) 1.7 – 2.

2.2. in vitro studies Calf thymus DNA and B[a]P

Samples of 50 µL (±5 µg) calf thymus DNA (100 µg/mL) were dissolved in phosphate buffer 0.1 M (pH 7.4 dan 8.5) reacted with 50 µL (±50 µg) benzo[a]pyrene (1000 µg/mL) at either 37°C and 60°C for 2 h.

2.3. in vitro studies Calf thymus DNA and $CuCl_2$

Samples of 50 µL (±5 µg) calf thymus DNA (100 µg/mL) were dissolved in phosphate buffer 0.1 M (pH 7.4 dan 8.5) reacted with 50 µL $CuCl_2$ 1.2 x 10-2 M (170 µg/mL) at either 37°C and 60°C for 2 h.

2.4. *Calf thymus* DNA Hydrolisis

DNA mixtures after incubation, were sentrifuged. Filter was precipitated and hydrolyzed using *micrococcus nuclease* (MN) dan *spleen phosphodiesterase* (SPDE) enzymes (total ratio of enzymes 0.02 unit : 0.002 unit) in total volume mixture of enzymes 100 µL. The mixture of enzymes were added 133 µL sodium succinate 10mM pH 6 and calsium chloride 5mM. The mixtures were incubated for 3 h at 37°C. DNA-adducts 8-OHdG were analyzed using HPLC.

3. RESULTS

This study aims to provide scientific information on the effects of free radicals contributor compounds which trigger oxidative damage to DNA. There is some evidence showed that oxidative DNA damage is promutagenic and has potential as biomarker of cancer (Kasai, 1997). In vitro studies were conducted using purified calf thymus DNA at physiological pH and reacted with toxic compounds which have potential in the formation of free radicals such as benzo[a]pyrene (B[a]P), and $CuCl_2$.

The measurement of absorbance ratio using spectrophotometers UV-Visible will measure any molecules absorbing at a specific wavelength. Samples of DNA will require purification prior to measurement to ensure accurate results. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA. A ratio 1.7 – 2 is generally accepted as "pure" for DNA.

$$\lambda 260 = 0.042$$

$$\lambda 280 = 0.022$$

$$\lambda 260/ \lambda 280 \sim 1.9$$

Both DNA and RNA absorb maimally at 260 nm, while most proteins absorb strongest at 280 nm. Nucleic acids also absorb signifivantly at 280 nm (50%-55% of the absorbance at 260 nm), and most proteins can absorb strongly at 260 nm (the absorbance varies, dependiing on the protein). Thus, it can be difficult to accurately measure the concentrations of DNAm RNA and protein in complex mitures. However, measuring absorbance at 260 nm and 280 nm can provide validation of the purity of nucleic acid samples: λ260/ λ280 ratios above 1.8 for DNA or 2.0 for RNA indicate pure samples; lower ratio values indicate the presence of protein or other contaminants (Gallagher,S.R. 1989).

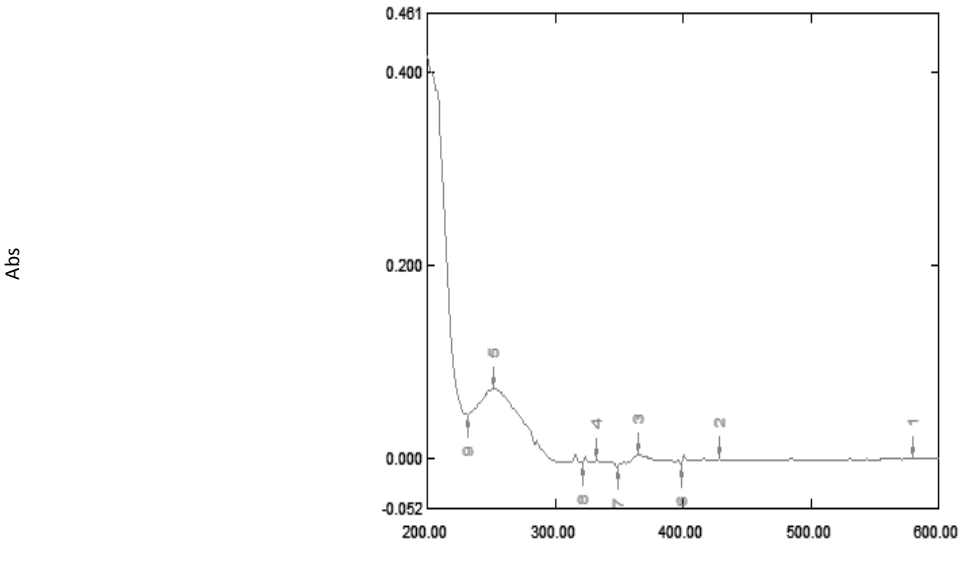


Figure 3.1. DNA spectrum profile

3.1. DNA Hydrolysis

Calf thymus DNA hydrolysis with MN and SPDE enzymes has done by Gupta, 1996. Ratio of DNA:enzymes was regulated based on incubation time, the ratio used for DNA hydrolysis is 1: 3 (enzyme: DNA) with incubation time for ± 3 hours, and 1 : 10 (enzyme: DNA) for ± 24-hour incubation period. Ratio of enzyme MN: SPDE used in this experiment was 10: 1 (20 Units / mL MN: 2 Units / mL SPDE) in 100 mL solution for DNA 100 ug / mL. The solution of enzyme hydrolysis $CaCl_2$ and sodium succinate were used with a concentration ratio of sodium succinate: $CaCl_2$ is 2: 1. This solution is used for MN: SPDE enzymes activation during DNA hydrolysis process. Volume ratio enzyme:hydrolysis solution is 3: 4. DNA hydrolysis with enzymes was done at 37°C for 2 h. Peak of DNA (100 µg/mL) hydrolysis with MN: SPDE enzymes is shown in Figure 3.2. The standard of 8-OHdG 100 ppb was spiked to DNA hydrolysis solution. Chromatograms standard 8-OHdG concentration of 100 ppb retention time results at 3.8 minutes. Based on literature, on the same condition and the same tools peak around retention time 2.8 minutes is deoxiguanosine (dG) (Rachmawati, 2014). 8-OHdG compounds analyzed in this experiment were detected in the samples and calculated as 8-OHdG concentration in ppb. Detection of 8-OHdG in the samples indicate that the compound using in this experiment which have high toxicity such as B[a]P, and $CuCl_2$, have a contribution to the formation of reactive oxygen species ($HO^{\bullet}$). Hydroxyl radical ($HO^{\bullet}$) which is formed can attack the C-8 position of guanine bases in DNA to form DNA adducts, 8-OHdG (Figure 3.1).

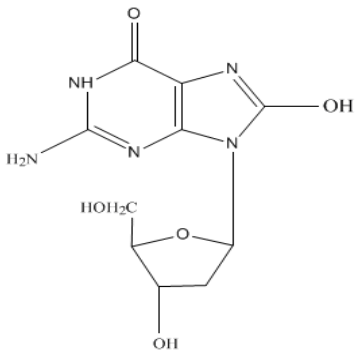


Figure 3.2. The structure of 8-hydroxy deoxiguanosine (8-OHdG)

3.2. Calf Thymus DNA Incubation with Compounds Trigger to Free Radicals (B[a]P and $CuCl_2$)

Calf thymus DNA incubation with compounds trigger to free radicals such as B[a]P and $CuCl_2$ was done with a variety of conditions, such as pH variations.

Based on human physiological pH (7.35-7.45), the pH experiments has been chosen at pH 7.4 and for comparison, pH 8.5 has been chosen as higher pH condition to see the profile of 8-OHdG adduct formation. Another variation of conditions are temperature variations. The temperature used was 37°C which is human physiological temperature and expected to describe the condition of human body which can occur various metabolic reactions. Moreover temperature of 60°C has been chosen, as comparison to prove the theory of the rise in temperature causes more quickly reaction. So that, the profile of adducts formation could be seen as impact of temperature rising.

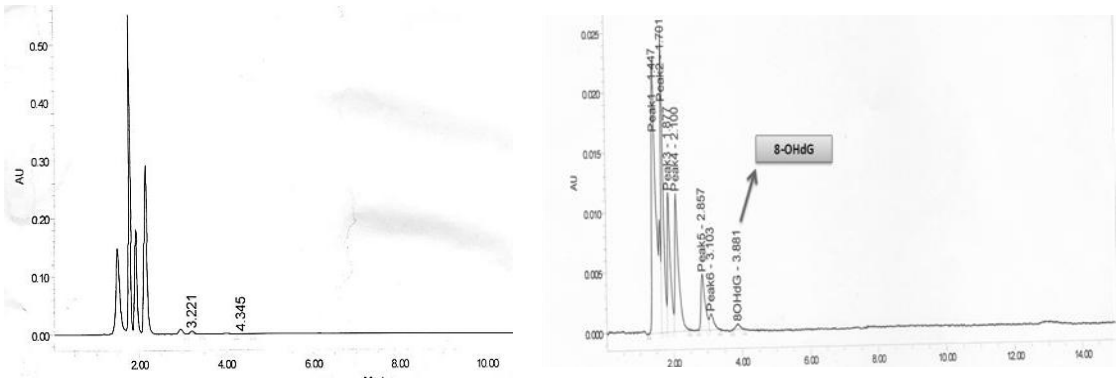
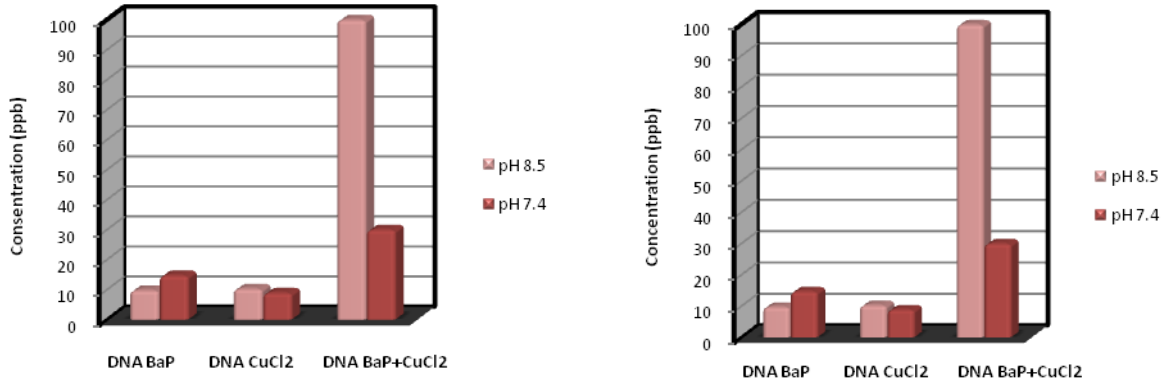


Figure 3.3. *Calf thymus* DNA hydrolysis chromatogram (a) and spike 100 ppb 8-OHdG (b)

a. The Effect of Compounds Trigger to Free Radical (B[a]P and $CuCl_2$) on the DNA adducts (8-OHdG) Formation

Incubation of calf thymus DNA results in phosphate buffer pH 7.4 and 8.5 with a compound B[a]P and $CuCl_2$ at 37°C and 60°C can be seen in Chart 3.1. Calf thymus DNA was incubated with each of B[a]P and $CuCl_2$, followed by incubation of calf thymus DNA with combination of B[a]P and $CuCl_2$. DNA adduct formation (8-OHdG) of incubation results indicate that the formation of 8-OHdG is occured because of B[a]P and $CuCl_2$ exposure. Both compounds are capable to trigger the formation of DNA adducts 8-OHdG synergetically.

Calf thymus DNA incubation at 37°C and pH 7.4 was produced 8-OHdG level. The results were 14.67 ppb for incubation with B[a]P and 8.86 ppb for incubation with $CuCl_2$, and the mixture of two compounds (B[a]P and $CuCl_2$), can be mutually synergistic forming DNA adducts 8-OHdG, a total of 8-OHdG level was 29.9 ppb (Data are not shown).



a. b.

Chart 3.1. The Effect of Compounds Trigger to Free Radicals (B[a]P and $CuCl_2$) on the Formation of DNA adducts (8-OHdG). a) Temperature 37 ° C b) Temperature 60 ° C

Calf thymus DNA incubation with B[a]P and $CuCl_2$ at 37°C and pH 8.5 in detail is shown in Chart 4.2 (b). Results showed that incubation of calf thymus DNA with B[a]P and $CuCl_2$ at pH 8.5 contributes to the formation of 8-OHdG.

Adduct results are not much different from the results under pH 7.4. Incubation calf thymus DNA and B[a]P generated 8-OHdG 9.7 ppb and 10.2 ppb after incubation with $CuCl_2$. The formation of adducts in both compounds incubation, providing a significant increasing in concentration compared with incubation in pH 7.4. Incubation of calf thymus DNA with a compound B[a]P and $CuCl_2$ at pH 7.4 and 8.5 was also performed at temperature 60°C. DNA adduct 8-OHdG formation profile at incubation temperature 60°C provide the same trend compared with incubation temperature at 37°C. Incubation of calf thymus DNA with B [a] P and $CuCl_2$ at 60°C in both pH 7.4 and 8.5 resulted in higher formation of 8-OHdG adducts than incubation of calf thymus DNA at 37°C, because the higher temperature, the more speed of reaction occured. At temperature 60°C, both pH 7.4 and 8.5 were incubated with B[a]P and $CuCl_2$ and those two compounds reaction synergetically occured to form DNA adducts (8-OHdG). The chromatogram profile of HPLC for calf thymus DNA incubation with B[a]P pH 8.5 and temperature 60°C shows the formation of 8-OHdG adducts is about 11 366 ppb (Figure 3.3).

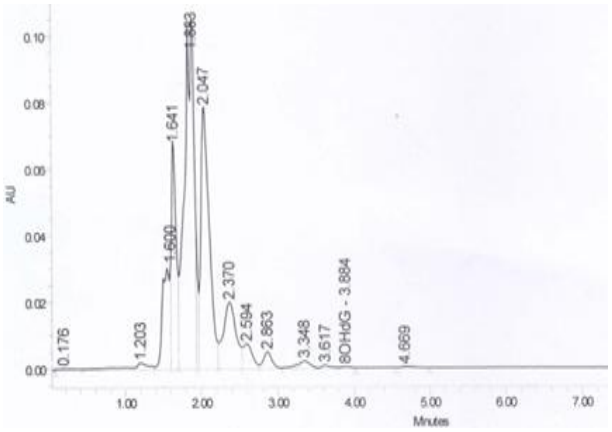


Figure 3.3. HPLC Chromatograms of Incubation Calf thymus DNA and Benzo[a]Pyrene pH 8.5 at 60°C

Some types of chemical reactions have been known to produce the formation of H_2O_2, $O_2^{\bullet-}$, atau $HO^{\bullet}$. Based on previous studies (Bryla, 1991), the hypothesis of the reaction is the oxidation of B[a]P during incubation with calf thymus DNA can causes the formation of H_2O_2, $O_2^{\bullet-}$, dan $HO^{\bullet}$ which can lead to binding B[a]P with DNA (B[a]P-adduct).

$$\rightarrow \qquad RH_2 + O_2 \qquad RH^{\bullet} + O_2^{\bullet-} + H^+$$

$$\rightarrow \qquad RH_2 + O_2 \qquad R + H_2O_2$$

In the reaction between the B[a]P and $CuCl_2$, there is role of Cu^{2+} as mediator ions which undergo redox cycle of Cu(II)/Cu(I). This reaction involves electron transfer that can lead to B[a]P oxidation. Haber-Weiss reaction principle using Fe catalyst, describes the reaction of hydroxyl radical formation.

Cu ions has potential to produce hydroxyl radicals by the Haber-Weiss reaction (Minotti, 1987). In addition, Fenton reaction also describes the formation $O_2^{\bullet-}$ and H_2O_2 in the presence of Cu^{2+} ions (Bodell, 1989).

$$Cu^{2+} + O_2^{\bullet-} \rightarrow Cu^+ + O_2$$

$$\rightarrow \qquad 2O_2^{\bullet-} + 2H^+ \qquad O_2 + H_2O_2$$

$$\rightarrow \qquad Cu^+ + H_2O_2 \qquad Cu^{2+} + OH^- + HO^{\bullet}$$

When Cu is not involve, reactions that may occur in the formation of hydroxyl radicals are:

$$\rightarrow \qquad O_2^{\bullet-} + H_2O_2 \qquad O_2 + OH^- + HO^{\bullet}$$

b. The Effect of Temperature on Formation of DNA adducts (8-OHdG)

In theory, an increase in temperature will speed up chemical reactions. This is proved by comparing the level of adducts at two incubation temperature 37°C and 60°C. Chart 4.3. shows the level of adducts resulted from calf thymus DNA incubation with B[a]P and $CuCl_2$ under pH condition 7.4. It describes the effect of temperature on DNA adduct formation (8-OHdG).

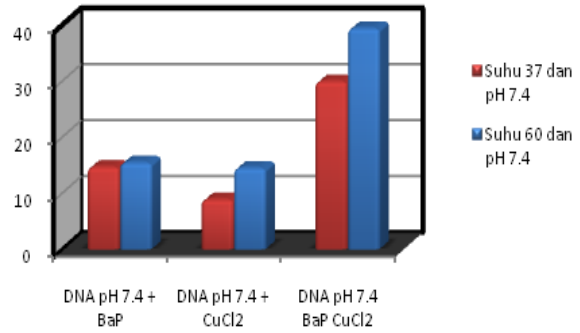


Chart 4.3. The Effect of Temperature on Formation of DNA adducts 8-OHdG at pH 7.4

Chart 4.3 showed the increasing of DNA adduct formation (8-OHdG) at incubation temperature 60°C. Similarly, the profile of DNA adducts formation (8-OHdG) is happened at conditions of pH 8.5. shown in Chart 4.4.

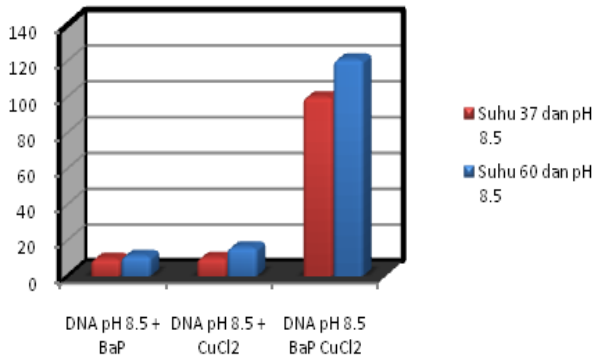


Chart 4.4. The Effect of Temperature on Formation of DNA adducts 8-OHdG at pH 7.4

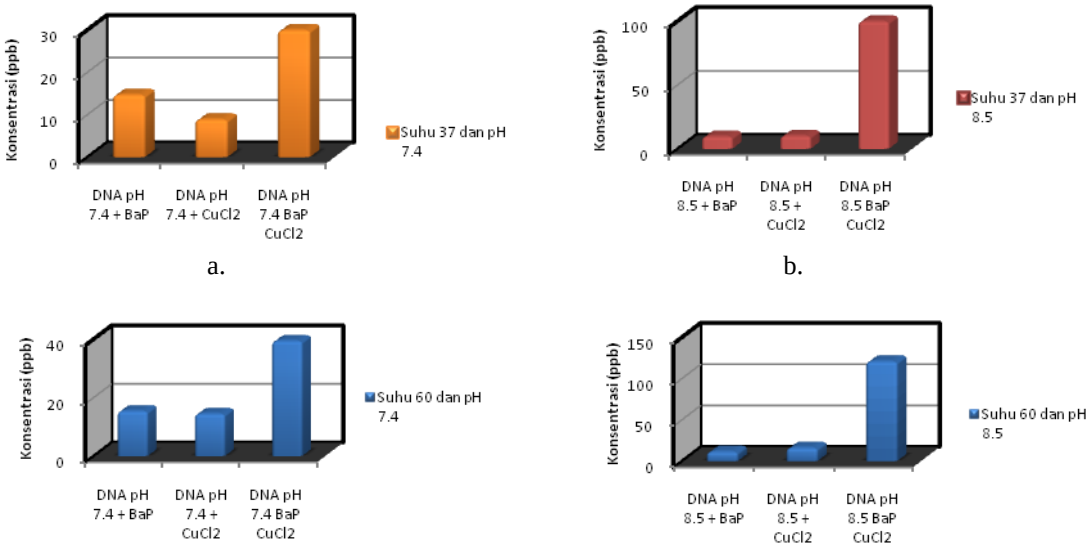


Chart 4.2. The effect of compounds trigger to free radicals (B[a]P and $CuCl_2$) in the formation of DNA *Adduct* (8-OHdG) a) temperature 37°C and pH 7.4, b) temperature 37°C and pH 8.5, c) temperature 60°C and pH 7.4, d) temperature 60°C and pH 8.5

4. CONCLUSION

1. Both compounds trigger to free radicals in this research (B[a]P and $CuCl_2$) has contribution in DNA Adduct formation (8-OHdG),
2. The formation of DNA Adduct in calf thymus DNA incubation with B[a]P is increasing by addition of $CuCl_2$ under condition of pH 7.4 dan 8.5,
3. The formation of DNA Adduct (8-OHdG) is higher at incubation temperature 60°C than 37°C.

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