In Vitro of Formation of 8-Hydroxy-2'-Deoksiguanosin (8 OHdG), in Calf thymus DNA and 2'-Deoksiguanosin Treated with Bisphenol A.

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Abstract

DNA damage due to oxidative processes can be analyzed by DNA adduct 8-hdroxy deoxyguanosin concentrat ion (8OHdG). The presence og 8-OHdG can be an indicator of cellular oxidative stress that may becoming biomarkers of DNA damage in the process of carcinogenesis. Bisphenol A and Fenton can stimulate oxydative stress to calf thymus DNA ang 2'deoxyguanosin that leads 8-OHdG forming. Its get in vitro testing through different condition, such as pH variation, temperature, concentration and incubation time. The result shown that BPA could potentially induced 8-OHdG forming with 1,8 purity ration (checked at λ260/ λ280). The different conditions also lead 8 OHdG forming concentration is higher in each variables (ranger between 4 – 70 ppb) than is control. .

Keywords: Bisphenol A, 8-hydroxy-2'-Deoksiguanosin (8-OHdG), Fenton reaction, DNA adducts.

Introduction

Plastic is a material that is easy to find in the market, because it is not easily broken and relatively cheaper, making it a popular choice. The most common ingredient for plastic is polycarbonate (PC). Polycarbonate plastic is used for various food and beverage packagings, baby bottles, food packages, medical equipments, dental sealants (thin layer of plastic used to cover the surface of teeth), CD, DVD, spectacle lenses and sports equipments as well as several paper coating (ATM receipts and cash counter receipts) (Gupta, Reproductive and Developmental Toxicology, 2011).

The main ingredient for making polycarbonate plastic is 2,2-bis (4-hydroxyphenyl) propane, also known as Bisphenol A (BPA). Aside from being the main material for polycarbonate (PC), BPA is also used as an epoxy resin material, mainly as inner coating on metal packaging products that serves to prevent corrosion, such as in food and beverage cans and water supply pipes. (International Food Safety Authorities Network (EFSA,2015)

BPA is carcinogenic - it induces cancer - because it interacts with DNA and causes what is known as DNA adducts due to mutations in DNA that can trigger continuous, out-of-control cell formation or division (Vineis, 2005). Carcinogenic compounds can trigger oxidative stress and contribute to the formation of reactive oxygen species (ROS). Upon entering the body, carcinogenic compounds will undergo a process called detoxification. It repairs damaged DNA through the mechanism of Excision Base Repair (BER) which can excrete cut-off damaged DNA. The DNA, which is damaged and truncated by BER, is found in the form of 8-hydroxy-2'-deoxyguanosine (8-OHdG) (Cooke et al., 2013).

Material and Method *Chemicals*

Calf thymus DNA, 2'-deoxyguanosine monohydrate, 8-OHdG, aquabidest, Bisphenol A, FeSO4, H2O2, phosphate buffer, acetate buffer, hydrochloric acid, sodium hydroxide, micrococcus nuclease enzyme (MN) and spleen phosphodiesterase (SPDE) enzyme, methanol , DMSO.

Analysis of 8-Hydroxy-2'Deoxyguanosine (8-OHdG) with HPLC

In vitro test was performed with calf thymus DNA and 2'-deoxyguanosine incubated with a BPA compound by adding fenton reaction performed on pH variation, temperature, concentration and incubation time, followed by observation of the produced 8-OHdG .

In Vitro Studies with Calf Thymus DNA and BPA

A total of ± 10 µg (in 100 µL) of calf thymus DNA (100 µg / mL) in 0.1 M of phosphate buffer (pH 7.4 and 8.4) is incubated with 10 µg (in 100 µL) of BPA solution in an incubator at 37 ° C and 37 ° C over a period of 6 hours.

In Vitro Studies with Calf Thymus DNA, BPA and H_2O_2

A total of ± 10 µg (in 100 µL) of calf thymus DNA (100 µg / mL) in 0.1 M of phosphate buffer (pH 7.4 and 8.4) is incubated with 10 µg (in 100 µL) of BPA solution in an incubator at 37 ° C over a period of 6 hours.

In Vitro Studies with Calf Thymus DNA, BPA, Fe(II) and H_2O_2

A total of ± 10 µg (in 100 µL) of calf thymus DNA (100 µg / mL) in 0.1 M of phosphate buffer (pH 7.4 and 8.4) is incubated with 10 µg (in 100 µL) of BPA solution, 249 µg of Fe (II) (in 50 µl) at 37 ° C over a period of 6 hours.

Hydrolysis of Calf Thymus DNA Enzymatically

Mixture of Calf Thymus DNA and Bisphenol A, the mixture of Calf Thymus DNA, Bisphenol A, FeCl2 and H2O2 and the mixture of Calf Thymus DNA , FeCl2 and H2O2 were centrifuged and the filtrate hydrolyzed using micrococcus nuclease enzyme (MN) and spleen phosphodiesterase (SPDE) enzyme (enzyme ratio 0.02 units: 0.002 unit) with a total enzyme mix of 100 μL. Added with 133 μL sodium succinate 10 mM pH 6 and calcium chloride 5 mM, then incubated for 3 and 9 hours at 37 ° C and 60 ° C, then analyzed using HPLC

In vitro study of 2'-deoxyguanosine and BPA 2 ppm and 10 ppm

100 μL 2'-deoxyguanosine (600 pbb and 3 ppm) in 0.1 M phosphate buffer solution with pH of 7.4 and 8.4 is added with 100 μL of BPA solution (6 ppm and 30 ppm). The sample is then added with aquabidest until final volume of 300 μL and incubated over a varying period of 3 and 9 hours at a varying temperature of 37 ° C and 60 ° C.

In vitro study of 2'-deoxyguanosine, BPA (2 ppm and 10 ppm) and H_2O_2

100 μL of 2'-deoxyguanosine (600 rpb and 3 rpm) in 0.1 M phosphate buffer solution with pH of 7.4 and 8.4 is added with 100 μL of BPA solution (6 ppm and 30 ppm) and 100 μL of H2O2 (6 ppm dan 30 ppm). The sample is then incubated over a varying period of 3 and 9 hours at a varying temperature of 37 ° C and 60 ° C.

In vitro study of 2'-deoxyguanosine, BPA (2 ppm and 10 ppm), Fe(II), and H_2O_2

100 μL of 2'-deoxyguanosine (600 ppb and 3 ppm) in 0.1 M phosphate buffer solution with pH of 7.4 and 8.4 is added with 100 μL of BPA solution (6 ppm and 30 ppm) and 50 μL of H2O2 (12 ppm dan 60 ppm). The sample is then incubated over a varying period of 3 and 9 hours at a varying temperature of 37 ° C and 60 ° C.

Result and Discussion

Result of 8-OHdG DNA Adduct Creation with 2'Deoxiguanosine

Carcinogenic compounds, upon attacking DNA, can cause DNA damage due to its contribution to the formation of reactive oxygen species (ROS). If the mechanism of DNA repair in the body is slower than the rate of DNA damage, mutations will occur, eventually leading to the onset of cancer. The hydroxyl radical (OH) formed by the ROS mechanism can attack the guanine base in DNA to form an 8-OHdG DNA adduct. 8-OHdG is a DNA damage produced by the addition of hydroxyl radical at C-8 guanine position to the DNA.

Variation of reaction, Incubation time 3 hours, BPA 2 ppm	Concentration 8-OHdG (ppb)			
	Temperature 37^0 C		Temperature 60^0 C	
	pH 7,4	pH 8,4	pH 7,4	pH 8,4
dG + BPA	3,5 79	6,6 72	6,5 33	3,7 86
dG + BPA + H_2O_2	3,6 94	6,9 48	9,3 03	14, 751
dG + BPA + Fe(II) + H_2O_2	10, 849	12, 950	11, 219	12, 953

Variation of reaction, Incubation time 9 hours, BPA 2 ppm	Concentration 8-OHdG (ppb)			
	Temperature 37^0 C		Temperature 60^0 C	
	pH 7,4	pH 8,4	pH 7,4	pH 8,4
dG + BPA	10, 780	11, 080	5,4 71	7,1 56
dG + BPA + H_2O_2	6,9 71	14, 635	4,7 51	7,8 26
dG + BPA + Fe(II) + H_2O_2	15, 997	8,2 64	6,9 71	15, 859

Variation of reaction, Incubation time 9 hours, BPA 2 ppm	Concentration 8-OHdG (ppb)			
	Temperature 37^0 C		Temperature 60^0 C	
	pH 7,4	pH 8,4	pH 7,4	pH 8,4
dG + BPA	7,7 79	8,4 26	14, 127	15, 536
dG + BPA + H_2O_2	9,9 26	10, 134	7,3 87	15, 951
dG + BPA + Fe(II) +	15,	6,1	15,	19,

H_2O_2	351	63	651	298

Variation of reaction, Incubation time 9 hours, BPA 2 ppm	Concentration 8-OHdG (ppb)			
	Temperature 37^0 C		Temperature 60^0 C	
	pH 7,4	pH 8,4	pH 7,4	pH 8,4
dG + BPA	6,187	20,614	14,635	21,676
dG + BPA + H_2O_2	9,118	26,223	17,221	40,212
dG + BPA + Fe(II) + H_2O_2	20,845	24,077	39,098	39,151

Result of 8-OHdG DNA Adduct Creation with 2'Deoxiguanosine

Carcinogenic compounds, upon attacking DNA, can cause DNA damage due to its contribution to the formation of reactive oxygen species (ROS). If the mechanism of DNA repair in the body is slower than the rate of DNA damage, mutations will occur, eventually leading to the onset of cancer. The hydroxyl radical (OH) formed by the ROS mechanism can attack the guanine base in DNA to form an 8-OHdG DNA adduct. 8-OHdG is a DNA damage produced by the addition of hydroxyl radical at C-8 guanine position to the DNA.

Temperature Effects

Result of incubation of 2'Deoxyguanosine with BPA, H2O2 and the addition of fenton reagents at temperatures of 37 ° C and 60 ° C. In chemical reaction / synthesis, based on the principle of kinetics each temperature increase (10 ^{O}C) will in turn increase the amount of the products created by the the reaction / synthesis, but it will not change the compound / product created at a certain temperature increase. Therefore, based on the research, most samples treated at 60 ° C have a greater concentration of adducts than at 37ºC. The use of 60°C temperature in this experiment is also to anticipate further need to analyze the characteristic of adduct (product of reaction) to obtain just the amount required to characterize adduct compound at 60 ^{O}C without damaging possible DNA Adduct compound.

pH Effects

pH 7.4 and 8.4 were used for the test. pH 7.4 is used because it's in accordance with the physological pH of the human body (7.35 - 7.45), whereas pH 8.4 is used to compare the result of DNA adduct 8-OHdG obtained at a higher pH (alkaline). Furthermore, for research purposes, if adduct characterization is required (product of reaction), the amount/ dosage required is enough to characterize a number of adducts produced at alkaline pH (8.4) without damaging the possible desired DNA adduct compound. The results of the research indicates that the 8 OHdG levels formed are largely higher at pH 8.4 than pH 7.4.

Effect of Incubation Time

The effect of incubation time on the formation of 8-OHdG, where the longer incubation time the more DNA Adduct 8 OHdG will be formed in accordance with the theory where the reaction rate will increase along with the length of contact time. The longer the incubation time

is used, the longer and more often a molecule undergoes a collision. 3 and 9 hours of incubation time are used. The DNA Adduct 8 OHdG production rate is higher for 9 hours of incubation time than for 3 hours.

Effect of BPA Concentration

Varied BPA concentration of 2 and 10 rpm are used to identify the relationship between the formed 8-OHdG concentration and the BPA concentration in the sample. Based on the results of this study, the larger the concentration of BPA the higher the amount of 8-OHdG concentration. According to this theory, most 8-OHdG compounds are formed at BPA concentration of 10 rpm. This is Because the BPA acts as pro-oxydant. BPA can increase the formation of hydroxil radicals which can bind with the DNA to form DNA Adduct.

Result of 8-OHdG DNA Adduct Formation with Calf thymus DNA

Incubation of Calf thymus DNA with BPA

The formation of DNA adducts from incubation with this HPLC-analyzed BPA compound detects the formation of 8 OHdG at retention time of 9.386 (Fig. 1) with a concentration of 8 OHdG of 2.580 ppb, whereas at retention time 6,817 it's the peak of deoxyguanosine (dG). This result is based on dG standard ad 8 OHdG which was previously analyzed under similar equipment condition.

The formation of 8 OHdG DNA Adduct in the sample indicates that BPA may contribute to the reactive oxygen species (ROS) that are hydroxyl radicals which bind with deoxyguanosine to form 8 OHdG (Sakuma, et al., 2010).

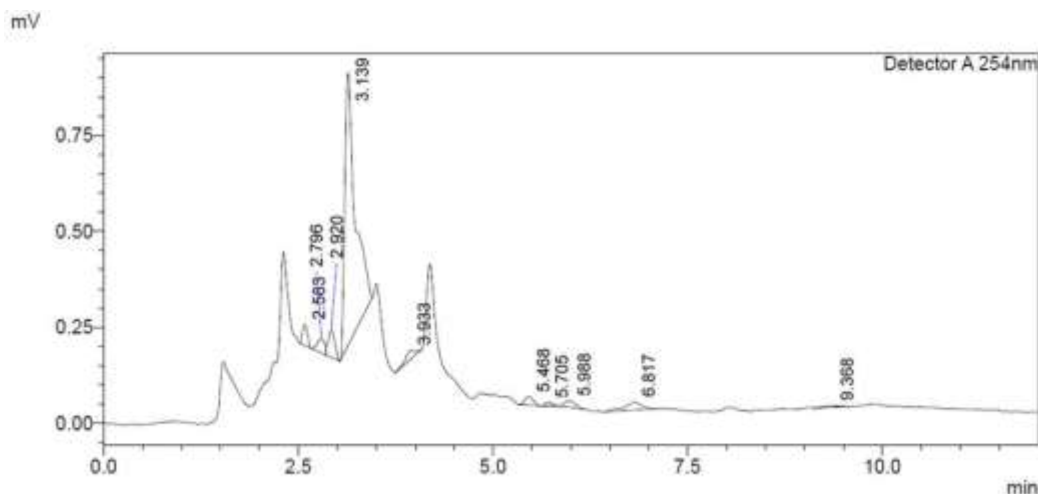

Fig. 1. HPLC chromatogram on the result of hydrolysis between the calf thymus DNA and BPA

Incubation of Calf thymus DNA with BPA and H_2O_2

The chromatogram shows that incubation with BPA and H_2O_2 compounds is capable of contributing to the formation of 8-OHdG. The resulting 8-OHdG concentration is 6.950 ppb at retention time of 10.067 and at retention time 7.012 it is deoxyguanosine (dG).

Based on the results of this test, the reactive exposure of oxygen species does not only come from BPA compounds, but also the addition of H_2O_2. H_2O_2 itself is one of the major reactive oxygen species (ROS) among organisms that are the result of metabolic process. The formation of H_2O_2 in the body through the action of superoxide dismutase (SOD) enzyme occurrs through the following mechanism:

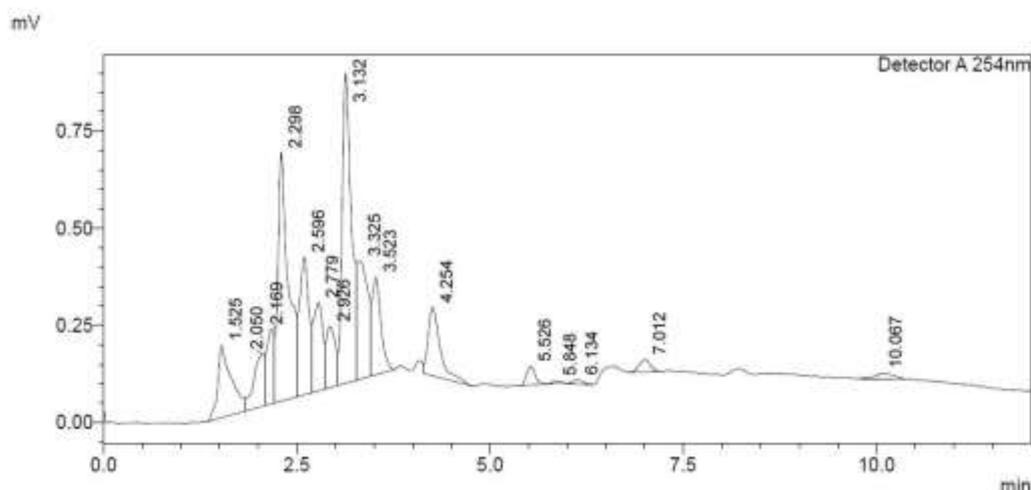

Fig. 2. HPLC chromatogram on the result of hydrolysis between the calf thymus DNA and BPA and H_20_2

Incubation of Calf thymus DNA with BPA, Fe(II) and H_2O_2

Test result is the formation of 8 OHdG at retention time of 10.132. HPLC chromatogram of incubated calf thymus DNA at pH 7.4 and 37°C temperature shows 8-2 OHdG yield of 7.272 ppb, whereas peak deoxyguanosine appears at retention time of 7.030 (Fig. 3)

Fenton reagents (Fe (II) metal and H_2O_2) in this variation are used to determine the effect of fenton reagents in the formation of 8 OHdG. Based on the results, the amount of 8 OHdG is higher than just with the addition of BPA and H_2O_2. The reactive oxygen species

produced from this fenton reagent bound to dG forms 8-OHdG through the following mechanism:

In the human body, the fenton reaction takes place through the mechanism of P450 cytochrome. The mechanism of the fenton reaction itself is the reduction of hydrogen peroxide by transitional metal ions, producing reactive hydroxyl radicals and oxidized metal ions (Mwebi, Nixon Ogendi. 2005).

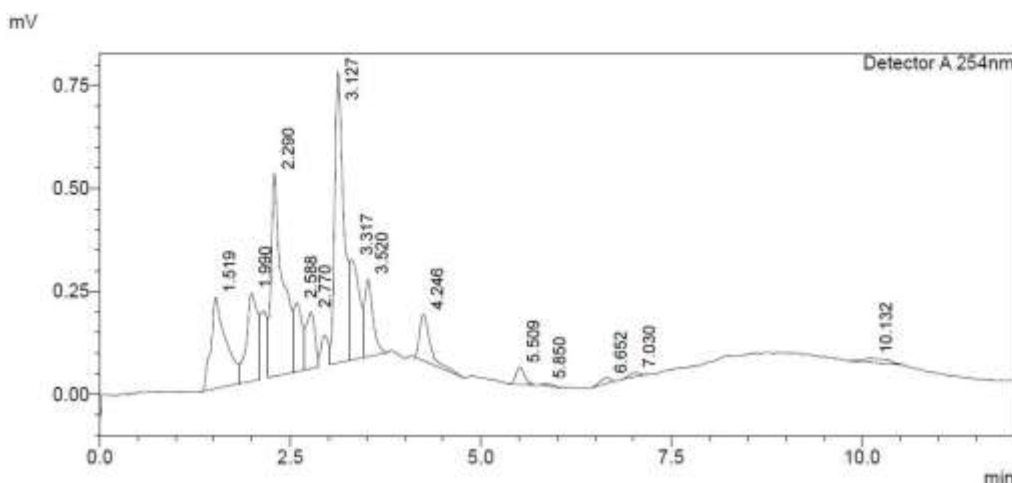

Fig. 3. HPLC chromatogram on the result of hydrolysis between the calf thymus DNA and BPA, Fe(II) and H_2O_2

Conclusion

1. Most of the samples have an 8-OHdG yield trend that will increase as the temperature, pH, concentration increase and incubation time become longer.
2. The reaction between dG and BPA may increase the concentration of 8-OHdG when in the presence of fenton reagents (Fe (II) and H2O2) in the reaction.
3. Incubation of calf thymus DNA with free radical contributor compound that is BPA with addition of fenton reagent can produce 8 OHdG equal to 7,272 ppb.

References

Gupta, R. C. (2011). *Reproductive and Developmental Toxicology.* London: Elsevier. 51

European Food Safety Authority (EFSA). (2015). Scientific Opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs: Executive summary. *EFSA Journal*.

Cooke, M. S., Evans, M. D., Dizdaroglu, M., and Lunec, J., 2003. Oxidative DNA damage:

mechanisms, mutation, and disease. The FASEB Journal, 1195-1214.

Sakuma, S., Nakanishi, M., Morinaga, K., Fujitake, M., Wada, S.-i., & Fujimoto, Y. (2010).

Bisphenol A 3,4-quinone induces the conversion of xanthine dehydrogenase into oxidase in vitro. *Food and Chemical Toxicology*, 2217-2222. Skoog, D. A., West, D. M., Holler, F. J., & Crouch, S. R. (2014). *Fundamentals of Analytical Chemistry.* Belmont: Mary Finch.

Sakuma, S., Nakanishi, M., Morinaga, K., Fujitake, M., Wada, S.-i., & Fujimoto, Y. (2010). Bisphenol A 3,4-quinone induces the conversion of xanthine dehydrogenase into oxidase in vitro. *Food and Chemical Toxicology*, 2217-2222. Skoog, D. A., West, D. M., Holler, F. J., & Crouch, S. R. (2014). *Fundamentals of Analytical Chemistry.* Belmont: Mary Finch.