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Original Research

Cytotoxicity study of benzo[a]pyrene on blood cells of Wistar rat in vitro

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Abstract: The effect of benzo[a]pyrene (BaP) on Wistar rat lymphocyte activity, lymphocyle membrane integrity and erythrocytes hemolysis were examined using the MTT assay, the release of the lactate dehydrogenase (LD) and the hemoglobin release respectively in vitro. The results showed that lymphocyte activity had no remarkable response to BaP after a low dose of exposure. But the membrane change was found after higher dose of BaP exposure, and the significant release of LD was observed. The release of LD increased with exposure time prolonging and exposure dose increasing. The results also showed that BaP didn't change the release of hemoglobin from erythrocytes. But the release of hemoglobin was affected significantly by synergistic effects of BaP and pentachlorophenol (PCP) and the release of hemoglobin increased with the dose of the mixture of BaP and PCP. Therefore, there is an obvious cytotoxicity of BaP in lower dose to blood cells of Wistar rat. The damage of lymphocyte membrane is more sensitive than lymphocyte activity and erythrocytes hemolysis to lower dose of BaP. It is suggested that the release of LD as a biomarker could be used to monitor and evaluate the contamination of BaP. The blood of Wistar rats were taken and checked after a fixed time interval (6 hours) of acute BaP exposure. The level of whole genomic DNA methylation was found to decrease significantly in the blood of rats.

Key words: Benzo[a]pyrene, cytotoxicity, lymphocyte activity, erythrocyte hemolysis, Wistar rat.

Introduction

Benzo[a]pyrene (BaP) is a polycyclic aromatic hydrocarbon with the formula $C_{20}H_{12}$, the compound is formed by a benzene ring fused to pyrene (1,2). BaP presents in coal tar, automobile exhaust fumes and all smoke (including cigarette smoke) resulting from the combustion of organic materials (3). BaP has been confirmed to have toxicity to nervous system, immune system, reproductive system, etc (4-6). A large number of studies over the previous two decades have reported the relationships between BaP and cancers. However, it has been more difficult to relate cancers to specific BaP sources, especially in human bodies, also it is difficult to quantify risks posed by various methods of exposure (i.e. inhalation or ingestion) (5-9). After BaP enters animal or human body, it interacts with blood cells and functional protein in blood through the blood circulation in the body and it can be transported to target organs (6,8,10,11-16). Therefore, a change in blood components is one of the early indicators for biological toxicity detection of chemical compounds, this method has been widely used (14-16). In this study, Wistar rats were used to establish the in vitro cytotoxicity experiment, the effects of low concentrations of BaP on the lymphocyte activity in blood, lymphocyte membrane integrity and erythrocyte hemolysis were determined to elucidate the low concentration of BaP on rats blood cell toxicity, and to explore the sensitivity of these cell toxicity indexes. This study is helpful to provide scientific data and efficient method for ecological risk assessment of BaP. The toxicity and carcinogenesis mechanisms of BaP from the perspective of epigenetics were preliminarily discussed in term of BaP concentration.

Materials and Methods

Experimental animals

SPF grade male Wistar rats (age: 4-6 weeks, weight: 180-220 g) were purchased from Animal Center of Dalian Medical University. The rats were divided into the experimental group and the control group with three rats in each group. Toxic experiments started after a week of normal feeding, all rats in both groups were acutely exposed to BaP (dose: 600 mg/kg by bodyweight) by oral administration. The blood samples of rats were collected after 6 hours of BaP exposure.

Chemical reagents and enzymes

Benzo[a]pyrene and pentachlorophenol (analytical pure) were purchased from Hangzhou Chemical Reagent Factory (Zhejiang, China). HPLC grade methanol, dimethyl sulphoxide and formic acid were purchased from Sigma-Aldrich (USA). Ammonium hydrogen carbonate, ammonium acetate, nuclease Pl, phosphodiesterase I were purchased from Sinochem (Shanghai) Co. Ltd. Alkaline phosphatase was purchased from Shijiangzhuang Biotechnology Co. Ltd. (Hebei, China).

PBS solution was prepared by dissolving NaCl (5 g), KCl (0.8 g), $Na_2HPO_4 \cdot 12H_2O$ (3.2 g) and NaH_2PO_4 (0.6 g) in 500 mL distilled water followed by 30 min of high-temperature sterilization.

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MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide] (>98%, purchased from Shanghai Gefan Biotech Co Ltd) was dissolved in PBS solution to prepare a concentration of 10 mg/mL for backup use after filtering and sterilization.

Lymphocyte activity was measured by MTT method, the test result was expressed by stress index (SI), which indicates the active effect of the lymphocytes of the experimenal Wistar rats.

The cell membrane integrity was determined by measuring the lactate dehydrogenase (LD) release of lymphocytes. The experimental results were expressed by relative release amount (R) of LD. There is no release effect of LD when L value is less than 10%, and the cell membrane integrity is considered intact.

Red cell toxicity was determined by erythrocyte hemolysis (hemoglobin release). The test results were expressed with the relative release of hemoglobin (H). There is no effect of hemoglobin release when H value is less than 10%, and no hemolysis of red blood cells.

Cells separation and purification

The separation of blood lymphocytes of rats was conducted by a syringe with heparin via taking the blood from vein, the blood sample was centrifuged and washed to prepare cell suspension. Culture medium was used to regulate cell concentration to 2×10^6 /mL, the sample was used to test the integrity of the lymphocyte activity test and lymphocyte cell membrane. Separation and purification of red cells were conducted by the following procedures. The blood sample taken from vein was added into a sterilized centrifugal tube for centrifugation and washing. Cell suspension was prepared by homogeneous mixing the solution with a straw and used for red blood cell toxicity test, the solution was used within 12 h after preparation.

DNA extraction and hydrolysis

K0512 DNA purification kit (Thermo Fisher Scientific) was used to extract DNA in the blood samples. The ratio of absorbance (A_{240}/A_{260}) of DNA in blood samples was between 1.3 and 1.6. In a three-neck round flask, DNA sample (2 μg) was dissolved in de-ionized water (10 μL), the solution was then immediately loaded into an ice bath followed by denaturation at 80 °C for 10 min. Ammonium acetate (pH = 4.8, 0.2 mol/L, 10 μL) and nuclease P1 (1.5 U/μL, 10 μL) were added. The mixture was reacted at 60 °C in a water bath. After 5 hours, ammonium bicarbonate (pH = 4.8, 0.2 mol/L, 10 μL) and phosphodiesterase (0.015 U/μL, 10 μL) were added. Alkaline phosphatase (0.15 U/μL, 10 μL) was added after the solution was continuously reacted for 5 h at 35 °C. The solution was reacted for an extra hour at 25 °C to yield the final product. The completely hydrolyzed DNA samples were finally stored at -30 °C for analysis.

Data analysis

The experimental data were processed by SPSS 13.0 statistical software, and t test was conducted among the samples. Compared with the blank control group, $P<0.05$ was considered significant difference, $P>0.05$ indicates that there are no significant differences between the data.

Results

Effect of BaP on lymphocyte activity

The effect of low BaP concentration on Wistar rat lymphocyte activity is shown in Figure 1. Compared with the control group, the concentration has no significant effect on lymphocyte activity within the concentration range of 1-500 μg/L, as SI values show no significant changes.

Effect of BaP on release of lactate dehydrogenase in lymphocytes

The effect of low BaP concentration on release of lactate dehydrogenase in lymphocytes is shown in Figure 2. Under the conditions of different exposure time (1 h and 2 h) respectively, the relative amounts of released LD lymphocytes indicate increasing trends as BaP concentration increases. Moreover, the release amount of LD increases significantly ($P<0.05$) to the higher concentration group (500-1,000 μg/L), the L values are greater than 10%. With an extension of exposure time, the release amount of LD also increases significantly to the higher concentration group, while L values were less than 10% to the low concentration group (1-500 μg/L) with no significant effect.

Effect of BaP on the hemolysis of red blood cells

The effect of low BaP concentration on Wistar rats'

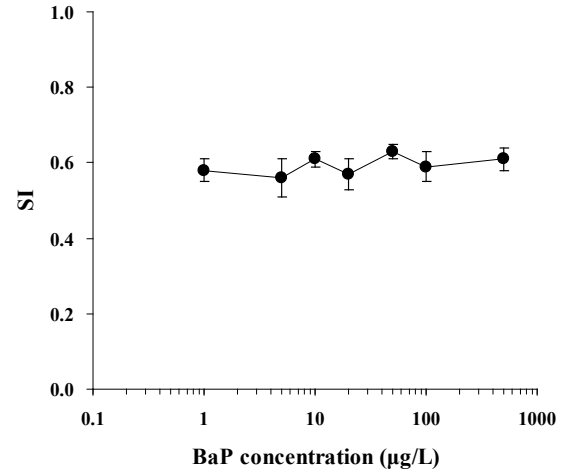

Figure 1. Effects of low concentration BaP on lymphocyte activity.

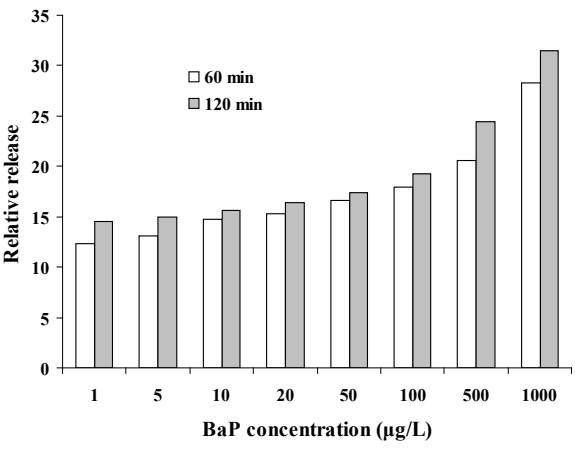

Figure 2. Effects of different BaP concentrations on the relative release of LD in different exposure time.

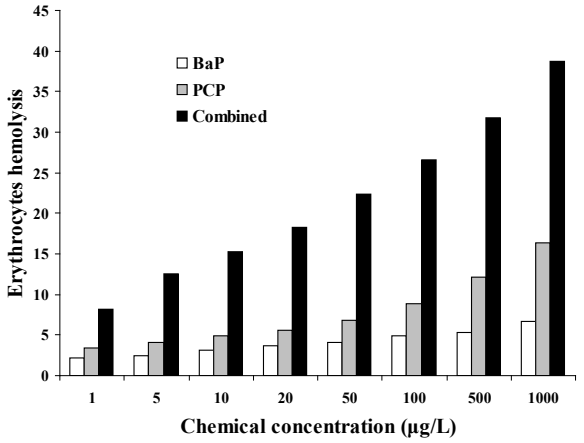

Figure 3. Effects of different chemicals on erythrocytes hemolysis in different exposure time.

erythrocyte hemolysis is shown in Figure 3. This result shows that BaP at low concentrations has no significant effect on rats erythrocyte hemolysis, the H values from the groups having various concentrations are less than 5%. Pentachlorophenol (PCP) at low concentrations (1-500 μg/L) has also no significant effect on rats' erythrocyte hemolysis. However, at higher concentrations (500-1,000 μg/L), PCP results in a significant effect on erythrocyte hemolysis ($P<0.05$), H values are all larger than 12%, and further increases dramatically with increasing concentration. The combined effect of BaP and PCP is significant, except for the groups with extremely low concentrations (1 and 5 μg/L) all other groups exhibit significant effects on hemolysis of red blood cells, H values are larger than 15%, and increases significantly with increasing chemical concentration.

Effect of acute exposure to BaP on DNA methylation in blood of Wistar rats

In order to confirm whether BaP exposure could lead to changes in the level of DNA methylation, in this study a high dose of BaP was administrated to the rats, as a method of acute exposure to BaP. During BaP exposure, the body weight of rats was not significantly changed, nor did the rats exhibit obvious abnormal behaviors.

Figure 4 shows the changes of the total genomic DNA methylation levels in the blood of rats over observation time after acute BaP exposure. As can be seen from the figure, the levels of DNA methylation in blood exhibits a downward trend compared with the blank group. For the exposed group, the levels of DNA methylation in blood significantly decreased ($p<0.05$) at some points of observation time. Blood DNA methylation level decreased to 78.8% of the blank group after 12 h of BaP exposure, and after 24 h, blood DNA methylation level decreased to 56.2% ($p<0.05$) of the blank group.

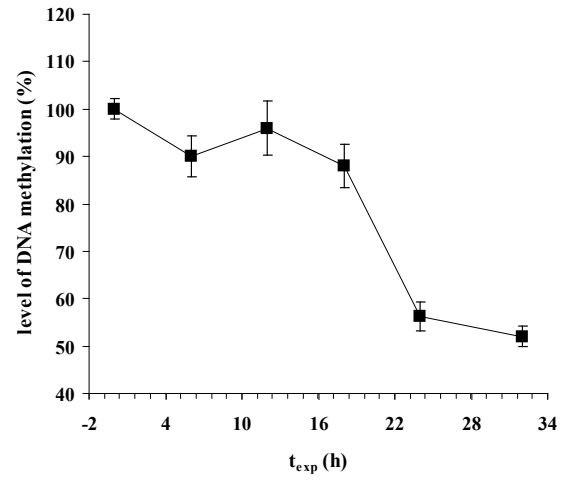

Figure 4. Changes of DNA methylation levels versus time in blood of dosed Wistar rats.

Discussion

BaP at low concentration (1-500μg/L) has no effect on rats lymphocyte activity and the hemolysis of red blood cells, but has significant effects on the cell membrane integrity at higher concentration (500-1,000 μg/L), the relative release amounts of LD significantly increase. The primary way that some chemical compounds result in the cell membrane damage is the change of permeability of cell membrane (11,12). Therefore, one of the possible mechanisms that cause damage to cell membrane integrity by BaP is the change of permeability of cell membranes, which leads to the release of lactate dehydrogenase (13). However, further experiments and studies should be conducted to provide the evidences that the permeability of cell membrane is changed by embedding lipophilic BaP onto the membrane lipid layer or by changing the permeability of ion channels in cell membrane. This study also found that the same chemical compounds caused different damage to different cell membranes. BaP at low concentration has no significant effect on erythrocyte membrane because no red blood cell hemolysis occurred by significant release of heme, probably due to different components of cell membrane. The red blood cells have intact membrane skeleton network which is closely connected with the cell membrane through the anchor protein and band protein to ensure the good elasticity and high strength of erythrocyte membrane.

BaP alone can produce biological toxicity, and can further corporate with other chemical compounds to generate much greater toxicity to organisms. This study exhibited that low concentration of BaP does not produce hemolytic effects, and the toxicity is far less than PCP. However, when combined with PCP, BaP made the red cell hemoglobin release increase significantly. This synergistic effect indicated that BaP presents more biological hazards in a real environment. But the mechanisms for this effect are not very clear.

As a pollutant, BaP commonly exists in the environment, BaP exposure to human body may cause skin cancer, lung cancer and other diseases (1-5). However, the mechanisms of the causation of diseases by BaP have not been completely revealed. Recently, some researchers have confirmed that the change of DNA methylation pattern may be the pathogenic mechanisms of some environmental carcinogens (11). In this study, the experimental results showed that BaP exposure can change the level of Wistar rats' DNA methylation. Blood is a target for the effects of BaP on the DNA methylation.

At lower concentration in the range of 1-500μg/L, BaP does not affect lymphocyte activity, but at higher concentration in the rang of 500-1,000 μg/L, BaP is cytotoxic, it can cause the damage of cell membrane, resulting in the release of LD. In the test range of concen-

tration, BaP has no significant effect on the hemolysis of red blood cells, but in can combine with PCP to produce synergistic effect, increasing toxicity to red blood cell. Cell membrane integrity is sensitive to low concentrations of BaP. Acute BaP exposure can change the DNA methylation level in rats, the level of DNA methylation decreases significantly in blood.

References

1. Harris CC, Vahakangas K, Newman MJ, Trivers GE, Shamsuddin A, Sinopoli N, et al. Detection of benzo[a]pyrene diol epoxide-dna adducts in peripheral blood lymphocytes and antibodies to the adducts in serum from coke oven workers. Proc Natl Acad Sci U S A 1985; 19: 6672-6.

2. Ross J, Nelson G, Erexson G, Kligerman A, Earley K, Gupta RC, et al. Dna adducts in rat lung, liver and peripheral blood lymphocytes produced by i.p. administration of benzo[a]pyrene metabolites and derivatives. Carcinogenesis 1991; 12: 1953-5.

3. Grova N, Salquèbre G, Hardy EM, Schroeder H, Appenzeller BM. Tetrahydroxylated-benzo[a]pyrene isomer analysis after hydrolysis of dna-adducts isolated from rat and human white blood cells. Toxicol Lett 2014; 229: 183-91.

4. Jones MD, Rodgers-Vieira EA, Hu J, Aitken MD. Association of growth substrates and bacterial genera with benzo[a]pyrene mineralization in contaminated soil. Environ Eng Sci 2014; 31:689-97.

5. Turja R, Guimarães L, Nevala A, Kankaanpää H, Korpinen S, Lehtonen KK. Cumulative effects of exposure to cyanobacteria bloom extracts and benzo[a]pyrene on antioxidant defence biomarkers in gammarus oceanicus, (crustacea: amphipoda). Toxicon 2014; 78: 68-77.

6. Beal MA, Gagné R, Williams A, Marchetti F, Yauk CL. Characterizing benzo[a]pyrene-induced lacz mutation spectrum in transgenic mice using next-generation sequencing. BMC Genomics 2015; 16: 1-13.

7. Kranz J, Hessel S, Aretz J, Seidel A, Petzinger E, Geyer J, et al. The role of the efflux carriers abcg2 and abcc2 for the hepatobiliary elimination of benzo[a]pyrene and its metabolites in mice. Chem Biol Interact 2014; 224: 36-41.

8. Lee SK, Lee BM. Oxidation of erythrocyte protein and lipid, and hemolysis in rabbit red blood cells treated with benzo[a]pyrene or adriamycin. J Toxicol Environ Health 1997; 51:557-69.

9. Kiruthiga PV, Karutha PS, Pandima DK. Silymarin prevents the toxicity induced by benzo(a)pyrene in human erythrocytes by preserving its membrane integrity: an in vitro study. Environ Toxicol 2014; 29:165-75.

10. Vijayapadma V, Ramyaa P, Pavithra D, Krishnasamy R. Protective effect of lutein against benzo(a)pyrene-induced oxidative stress in human erythrocytes. Toxicol Ind Health 2014; 30:284-93.

11. Zhao L, Zhang S, An X, Tan W, Pang D, Ouyang H. Toxicological effects of benzo[a]pyrene on dna methylation of whole genome in icr mice. Cell Mol Biol 2015; 61:115-9.

12. Santacroce MP, Pastore AS, Tinelli A, Colamonaco M, Crescenzo G. Implications for chronic toxicity of benzo[a]pyrene in sea bream cultured hepatocytes: cytotoxicity, inflammation, and cancerogenesis. Environ Toxicol 2015; 30:1045-62.

13. Crowell SR, Hansondrury S, Williams DE, Corley RA. In vitro metabolism of benzo[a]pyrene and dibenzo[def,p]chrysene in rodent and human hepatic microsomes. Toxicol Lett 2014; 228:48-55.

14. Bo J, Gopalakrishnan S, Chen FY, Wang KJ. Benzo[a]pyrene modulates the biotransformation, dna damage and cortisol level of red sea bream challenged with lipopolysaccharide. Mar Pollut Bull 2014; 85:463-70.

15. Tung EW, Philbrook NA, Belanger CL, Ansari S, Winn LM. Benzo[a]pyrene increases dna double strand break repair in vitro and in vivo: a possible mechanism for benzo[a]pyrene-induced toxicity. Mutat Res 2014; 760:64-9.

16. Collin A, Hardonnière K, Chevanne M, Vuillemin J, Podechard N, Burel A. et al. Cooperative interaction of benzo[a]pyrene and ethanol on plasma membrane remodeling is responsible for enhanced oxidative stress and cell death in primary rat hepatocytes. Free Radical Biol Med 2014; 72C:11-22.