DNA Strand Breaks and DNA-protein Crosslinks of Mast Cell Induced by Butyl Benzyl Phthalate

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***Abstract*—This experiment was about the effect of Butyl benzyl phthalate on DNA strand breaks and the DNA-protein crosslinks in cells. Mouse mast cells cultured in vitro were exposed to different concentrations of BBP (0、4、20、100 μmol•L^{-1}) for 1h. Comet assay and KCl-SDS assay were used to measure the DNA strand breaks and DNA-protein crosslinks. Results showed that, all of the BBP treatment groups (4、20、100 μmol•L^{-1}) could cause DNA damage, compared with the control group. But, BBP could induce DNA strand breaks at the lower dose (4 μmol•L^{-1}), and could primarily induce DPC at the middle dose (20 μmol•L^{-1}) and the higher dose (100 μmol•L^{-1}).**

Keywords- butyl benzyl phthalate; DNA strand breaks; DNA-protein crosslinks;

I. INTRODUCTION

Phthalate esters (PAEs) are widely used in industrial chemicals, serving as important additives to impart flexibility in polyvinylchloride (PVC) resins, and other industrial applications include the manufacturing of cosmetics, insect repellents, insecticide carries, and propellants [1]. They have become widely diffused in the environment [2] because they are not chemically bound to the PVC polymer; up to 1% of all phthalates from PVC products seeps out into the environment each year [3]. The United States Environmental Protection Agency (USEPA) and its counterparts in several other countries have classified the most commonly occurring PAEs as priority pollutants and endocrine disrupting compounds. Phthalates are generally viewed as endocrine disruptors that interfere with hormone production, release, transport, metabolism, and binding actions [4] and contribute to the development of hormone-dependent cancers [5].

Butyl benzyl phthalate (BBP), one of the PAEs commonly found in floor covering, and also exists in adhesives, sealants, and vinyl foams. Based on its wide distributions, BBP is known to be one of the most important manmade pollutants and has become a high concern in public health. Nowadays, BBP has recently been shown to have obvious reproductive toxicity, teratogenic and embryo toxicity [6]. In recent years, studies have shown that some phthalates chemicals such as DEHP, MEHP have genetic toxicity [7, 8], which can be induced DNA damages: DNA strand breaks (DSB), DNA-protein crosslinks (DPC), but very little is known about the genetic toxicity of the BBP. The aim of this study was to assess the genotoxic effects of BBP and to primarily identify the mechanisms, by using mouse mast cells.

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II. MATERIALS AND METHODS

A. Experimental equipments and main reagents

Low temperature refrigerated centrifuge (Eppendorf-5415R), BH-2-fluorescence microscope (Olympus), 0.635 cm 450-megapixel digital camera (Nikon), F-4500 fluore- scence spectrophotometer (Hitachi), DYY-Ⅲ electrophoretic slot (Beijing ba yi instrument factory), DYY-11B electrophoretic instrument (Beijing ba yi instrument factory), 5410-homoiothermal CO_2 incubator (Napco).

BBP (Wuhan yi xin Co.Ltd, purity>99.0%), 10% formalin solution (Sigma), normal melting point agarose and low melting point agarose (Premega), lauroyl sarcosine sodium (Merk), proteinase K (Merk), Hoechst-33258 fluorescent dye (Sigma), calf thymus DNA (Sigma).

B. Cell culture and treatment with BBP

Mast cells carcinoma-derived cell lines (P815) were purchased from Shanghai Institute of Cell Biology cell bank of the Chinese Academy of Sciences. Cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum, and grown at 37 °C in a 5% CO_2 humidified environment. The cells were subcultured every two days.

After centrifuging the cell culture medium, the precipitates were suspended with ice-cold PBS. Then the cell suspension was adjusted to10^5～10^6 cells per milliliter, and divided into four equal groups: one control group and three experimental groups respectively exposed to the designed concentrations of BBP (4, 20, 100 μmol•L^{-1}) for 1h.

C. Method

The comet assay used to measure DSB in individual cells was essentially the same as that described previously [9]. The cell suspension suspended in 1% low-melting-point agarose were dropped onto a frosted slide precoated with a layer of 1% normal melting point agarose and coated with additional 0.8% normal melting-point agarose. Slides were placed on ice for 20 min until they solidified. Coverslips were then removed and slides were immersed in lysing solution (2.5 M NaCl, 100 mM

Na_2EDTA, 10 mM TrisBase, 10% DMSO, 1% Triton X-100, 1% Lauroyl sarcosine sodium, pH 10) for 2 h at 4 °C, in the dark. Then the slides were exposed in an electrophoresis box containing alkaline buffer (300 mM NaOH, 1 mM Na_2EDTA, pH 13) at 4 °C for 20 min to allow for DNA unwinding and then subjected to 25 min electrophoresis at 16V and 110 mA in the same alkaline buffer. After electrophoresis, the slides were rinsed with 400 mM Tris–HCl, pH 7.5 three times in 10 min and stained with 50 μl ethidium bromide (50 $\mu g \cdot ml^{-1}$ in water), and covered with the cover slips. The analysis image was screened by using a fluorescence microscope with a digit camera. One hundred of randomly selected Comet cells from each sample were analyzed with ocular-micrometer. The DSB was evaluated by measuring the percentage of tail DNA and the tail moment with the use of Sigma Plot Comet Analysis software 9.0.

The KCl-SDS assay was applied to detect BBP-induced DPC [10, 11] with some modifications. Cells were then lysed with 2% SDS solution with gentle vortexing. The mixture was heated at 65℃ for 10min and 2.5 M KCl in 10 mM Tris-HCl, pH 7.4, was added, followed by passing the mixture six times through a 1mL polypropylene pipette tip to favor shearing of DNA to a uniform length. The SDS-KCl precipitate was formed by cooling the samples on ice for 5 min and was collected by centrifugation at 10 000 rpm for 5 min in an Eppendorf microfuge. The supernatants containing the unbound fraction of DNA were collected in tubes with sign. The pellets (containing DPC) were washed three times by resuspending each time in 1mL washing buffer (0.1 M KCl, 0.1 mM EDTA, and 20 mM Tris-HCl, pH 7.4) followed by heating the sample at 65℃ for 10min, chilling on ice for 5 min, and centrifugating as before. The supernatants from each wash step were pooled with previous unbound fractions. The final pellet was resuspended in 1 mL proteinase K solution (0.2 $mg \cdot mL^{-1}$ solubled in wash buffer) and digested for 3h at 50 ℃. The supernatants were collected by centrifugation at 12000rpm for 10min. Supernatant (1 mL) containing the unbound fraction of DNA or 1mL supernatant containing DNA previously involved in DNA-protein crosslinks was then mixed with 1mL freshly prepared fluorescent dye Hoechst-33258 (400 $ng \cdot mL^{-1}$ soluble in 20 mM Tris-HCl), respectively, and then the tubes were allowed to stand for 30min in the dark. The fluorescence of the sample was then measured in a RF-5301PC fluorescence spectrofluorimeter with excitation wavelength at 350 nm and emission wavelength at 450 nm. The DNA content of the sample was determined quantitatively from a corresponding DNA standard curve generated using calf thymus DNA. The DPC coefficient was estimated as a ratio of the percentage of DNA involved in DPC to the percentage of DNA involved in DPC and unbound fraction of DNA. The dose-effect relationships were plotted with Origin 6.1 software.

III. Results and Analysis

A. DSB of mast cells induced by BBP

As shown in Figure.1, it demonstrated that BBP could induce DSB of mast cells. In the 4$\mu mol \cdot L^{-1}$ BBP group, the percentage of tail DNA and the tail moment were both significantly higher than the control groups ($p < 0.01$), resulting in the DNA fragments and mobility added; With the concentration of BBP further increase, the percentage of tail DNA and the tail moment gradually reduced. In the 100 $\mu mol \cdot L^{-1}$ BBP group, the tail moment was even significantly lower than control ($p < 0.05$), this may be due to some fracture of the DNA to form the DPC, so that the DNA phoresis were blocked, and mobility reduced.

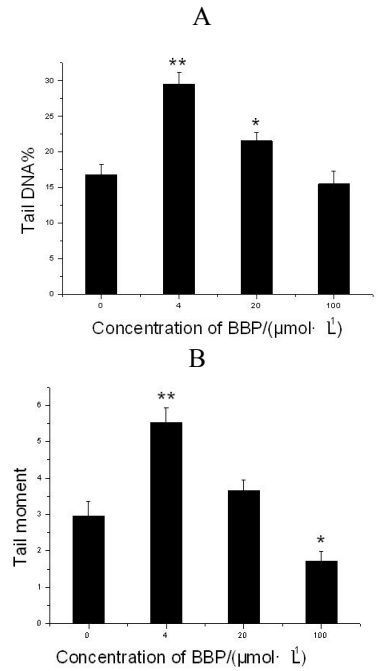

Figure 1. DSB in mouse mast cells induced by different concentration of BBP (A: Tail DNA %; B: Tail Moment; *, **: compared with the control group, $p<0.05$, $p<0.01$)

B. DPC of mast cells induced by BBP

As shown in Figure.3, there were no significant differences of DPC coefficients between the 4 $\mu mol \cdot L^{-1}$ BBP group and the control group ($p>0.05$), which suggested that low concentration of BBP would not lead to DPC; In the 20, 100 $\mu mol \cdot L^{-1}$ BBP groups, DPC coefficients were both obviously higher than the control groups ($p<0.01$). It demonstrated that low concentrations of BBP mainly caused DSB, and the high concentration of BBP could induce DPC significantly. The results were essentially the same as the comet assay results (Figure. 1).

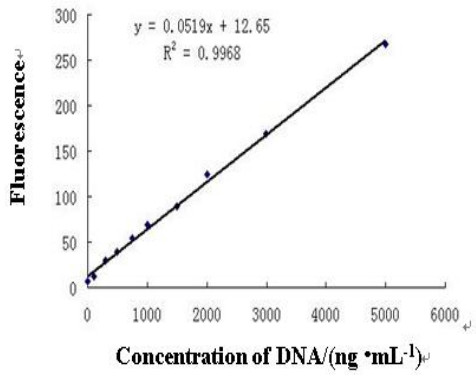

Figure 2. The standard curve of DNA concentration

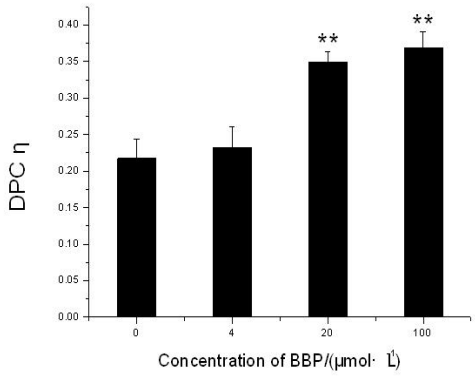

Figure 3. DPC in mouse mast cells induced by different concentration of BBP(**: compared with the control group, p<0.01)

IV. Discussion

In this study, we demonstrated that BBP could cause DSB and DPC in mouse mast cells on the molecular level by using the comet assay and the KCl-SDS assay. The results showed that all of the treatment concentrations (4、20、100 μmol•L^{-1}) of the BBP could cause DNA damage, low levels of BBP mainly caused DSB, and middle and high levles of BBP mainly caused DPC. Since the 1980s, more and more studies have concentrated on the genetic toxicity of PAEs, but mainly focused on DEHP [12], the studies on the genetic toxicity of BBP is still rare. Gaunt, et al treated the mouse embryonic fibroblast (3T6 cells) with different concentrations of DEHP (62.5、125、250、500 μg•mL^{-1}), which were found that with the increase of DEHP exposure concentrations, the DNA damage of 3T6 cells were aggravating, indicating a dose-response relationship [13]. Wu Kai, et al. exposed the mouse liver cells to DEHP, and found that low concentrations of DEHP could not cause DPC, but high concentrations of DEHP could cause DPC significantly [14], these were basically the same as the results of our study.

At present, the genetic toxicity mechanism of PAEs is inconclusive, most of the domestic and foreign scholars are apt to consider that PAEs indirectly induce genetic toxicity [15]. Theu acted on some cellular receptors to mediate their cytotoxicity. Some phthalates and their metabolites have the potential to cause carcinogenic hazards that might be related to their character to cause peroxisome proliferation through activating peroxisome proliferators-activated receptors and binding to peroxisome proliferators response elements [16]. In addition to this, some phthalates compete with estradiol for binding to estrogen receptors and induce estrogen receptor-mediated responses [17]. So we speculated that after BBP entered into the cells, it could act on the mitochondria [18], open the permeability transition pore, and inhibite the respiration of mitochondrial, resulting in the production of reactive oxygen species (ROS), and oxidative stress in cells fortified, thus DNA were fractured. ROS attacked on the bases, not only acted on the DNA backbone, but also acted on other components of cells to form more active bases through the chain reaction. They continued to attack DNA, gave rise to DSB and DPC, and ultimately leading to genetic toxicity [19].

Studies have shown that formaldehyde can generate similar genetic toxicity effects like BBP. Deng Yanmei, et al. found that low concentration of formaldehyde could cause DSB in P815 cells, and the high concentration of formaldehyde could cause DPC significantly; Such as Xu Xiaoyu also found that with increase of the formaldehyde exposure concentration, the DNA damages in spider cells were showed basically as DSB →DDC→DPC [20]. These clued that the effect of BBP may be similar to formaldehyde. BBP could produce hydroxyl radicals. Hydroxyl radicals are important factors to induce DPC: hydroxyl radicals can not only oxidate amino acids to generate a variety of intermediate products, but also directly link to DNA to form different types of base- modified products or cause the fracture of DNA chains. These finally induced DPC [21].

V. Conclusion

In conclusion, BBP at different concentrations could induce different DNA damages, leading to DSB at low concentrations and DPC at high concentrations.

References

[1] D. B. Peakall, "Phthalate esters: occurrence and biological effects," Residue REV, vol. 54, pp. 1–41. 1975.

[2] S. Jobling, T. Reynolds, R. White, M. G. Parker, J. P. Sumpeter, "A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic," ENVIRON HEALTH PERSP, vol. 103, pp. 582–587, 1995.

[3] R.N.K., Furtmann, "Phthalates in the aquatic environment, " EUR CHEM IND Council. Report vol 6/93, 1996.

[4] T. , Colborn, F.S., vom Sall, A.M., Soto, "Developmental effects ofendocrine-disrupting chemicals in wildlife and humans," ENVIRON. HEALTH PERSP, vol. 101, pp. 378– 384, 1993.

[5] D.L., Davis, H.L., Bradlow, M., Wolff, T.,Woodruff, D.G., Hoel, H., Anton-Culver, "Medical hypothesis: xenoestrogens as preventable causes of breast cancer," ENVIRON. HEALTH PERSP, vol. 101, pp. 372– 377. , 1993.

[6] A. H. Piersma, A. Verhoef, J. Te Biesebeek, M. N. Pieters, W. Slob, "Developmental toxicity of butyl benzyl phthalate in the rat using a multiple dose study design," REPROD TOXICOL, vol. 14, pp. 417–425, 2000.

[7] J. Doull, R. Cattley, C. Elcombe, B. G. Lake, J. Swenberg, C. Wilkinson, et al. "A cancer risk assessment of di (2-ethylhexyl) phthalate: Application of the new U. S. EPA risk assessment guideline," REGUL TOXICOL PHARM, vol. 29, pp. 327–357,1999.

[8] N. H. Kleinsasser, U. A. Harreus, E. R. Kastenbauer, B. C. Wallner, A. W. Sassen, R. Staudenmaier, et al., "Mono (2-ethylhexyl) phthalate exhibits genotoxic effects in human lymphocytes and mucosal cells of the upper aerodigestive tract in the comet assay," TOXICOL LETT, vol. 148, pp. 83–90, 2004.

[9] R. Gabbianelli, M. Villarini, G. Falcioni, G. Lupidi, "Effect of different organotin compounds on DNA of gilthead sea bream (Sparus aurata) erythrocytes assessed by the comet assay," APPL ORGANOMET CHEM, vol. 16, pp. 163–168, 2002.

[10] A. Zhitkovich, M. Costa, "A simple, sensitive assay to detect DNA - protein crosslinks in intact cells and in vivo," CARCINOGENESIS, vol. 13, pp. 1485–1489, 1992.

[11] S. K. Chakrabarti, C. J. Bai, K. S. Subramanian, "DNA-protein crosslinks induced by nickel compounds in isolated rat renal cortical cells and its antagonism by specific amino acids and magnesiumion," TOXICOL APPL PHARM, vol. 154, pp. 245–255, 1999.

[12] J. Doull, R. Cattley, C. Elcombe, B. G. Lake, J. Swenberg, C. Wilkinson, et al., "A cancer risk assessment of di(2-ethylhexyl) phthalate: Application of the new U. S. EPA risk assessment guideline," REGUL TOXICOL PHARM, vol. 29, pp. 327–357, 1999.

[13] I. Gaunt, K. Butterworth, "Autoradiographic study of orally administered di-(2-ethylhexyl) phthalate in the mouse," FOOD CHEM TOXICOL, vol. 20, pp. 215–217, 1982.

[14] K. Wu, L. M. Wang, D. D. Liu, S. M. Ding, X. Yang, Z.G. Xi, "Study on DNA- protein crosslinks of mice liver induced by di-(2-ethylhexyl) phthalate," J MED RES, vol. 11, pp. 13-15, 2006.

[15] J. E. Klaunig, M. A. Babich, K. P. Baetcke, J. C. Cook, J. C. Corton, R. M. David, et al., "PPARalpha agonist -induced rodent tumors: modes of action and humanrelevance," CRIT REV TOXICOL, vol. 33, pp. 655–780, 2003.

[16] C.H., Hurst, D.J., Waxman, "Activation of PPARalpha and PPARgamma by environmental phthalate monoesters," TOXICOL SCI, vol. 74, pp. 297–308, 2003.

[17] T.R., Zacharewski, M.D., Meek, J.H., Clemons, Z.F., Wu, M.R.,Fielden, J.B., Matthews, "Examination of the in vitro and in vivo estrogenic activities of eight commercial phthalate esters," TOXICOL SCI, vol. 46, pp. 282–293, 1998.

[18] W. L. Li, Y. B. Ji, Y. N. Yang, B. Yang, "Reproductive toxicity and functional mechanism of the environmental hormoune butyl benzyl phthalate," ENVIRON SCI, vol. 25, pp. 7–13, 2004.

[19] S. Dzhekova-Stojkova, J. Bogdanska, Z. Stojkova, "Peroxisomeproliferators: their biological and toxicological effects," CLIN CHEM LAB MED, vol. 39, pp. 468–474, 2001.

[20] X. Y. Xu, G. Q. Xiao, F. X. Liu, G. Y. Peng, Y. Peng, J. Chen, et al., "Genotoxicity of gaseous formaldehyde on the spider Eriovixia cavaleriei," J ASIAN ECOTOX, vol. 3, pp. 130–138, 2008.

[21] G. Y. Peng, Y. S. Liu, L. J. Duan, X. Yang, "DNA breakages and DNA crosslinks induced by formaldehyde," J PUBLIC HEALTH PREV MED, vol. 16, pp. 35–37, 2005.