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DNA damage in T- and B-lymphocytes of rats exposed to benzene

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***ABSTRACT*: Single cell gel electrophoresis assay was carried out to evaluate DNA damage in T-and B-lymphocytes from rats exposed to benzene and the correlation between DNA damage and the level of *t,t*-muconic acids, which are urinary benzene metabolites, was investigated. In control rats, the mean values of Olive tail moments in T- and B-lymphocytes were 1.507±0.187 and 1.579±0.206 respectively. DNA damages of T-lymphocytes in rats exposed for 4 weeks showed the highest Olive tail moments at each benzene concentration examined (2.72-4.351). However this DNA damage was decreased after 6 weeks of exposure (1.74-2.09). DNA damages of B-lymphocytes did not show such differences with exposure time or benzene concentration (1.49-2.07) except at 200 ppm at 4 weeks. T-lymphocytes show significantly more damages than B-lymphocyte upon acute exposure to benzene.**

Keywords : **comet assay, muconic acids, lymphocytes**

Introduction

Benzene is an aromatic hydrocarbon compounds which is volatile solvent used widely in industry and ubiquitous environmental contaminants as a component of cigarette smoke, gasoline, and automatic emissions (Paustenbach *et al.*, 1993; Kenyon *et al.*, 1996). The chronic exposure of humans to low levels of benzene in the workplace is associated with blood disorders including aplastic anemia and leukemia (Aksoy, 1985; Snyder *et al.*, 1977).

In animal, benzene has proven hematoxicity and it produces tumors in various tissues. Mice exposed to benzene showed malignant lymphoma, pulmonary adenoma, Zymbal grand squamous cell carcinoma, Harderian gland adenoma, and mammary gland carcinoma (Snyder *et al.*, 1980; Cronkite *et al.*, 1982; Huff *et al.*, 1989; Farris *et al.*, 1993; Farris *et al.*, 1997). Recently one of the mechanisms of benzene hematotoxicity has been clarified, in which benzene metabolites suppresses the cell cycle by p53-mediated overexpression of p21, a cyclin-dependent kinase inhibitor, resulting not simply in suppression of hemopoiesis but rather in a dynamic change of hemopoiesis during after benzene exposure (Yoon *et al.*, 2001).

Rozen and Snyder (1985) determined numbers of femoral B-lymphocytes, thymic T-lymphocytes, and splenic T- and B-lymphocytes in C57BL/6 mice exposed to 300 ppm of benzene. Farris *et al.* (1997) also evaluated the effects of low levels of benzene on femoral B-lymphocytes, thymic T-lymphocytes, and splenic T- and B-lymphocytes in B6C3F1mice. No significant effects on hematopoietic parameters were found for exposure to 10 ppm benzene or less.

Benzene metabolites induce apoptosis (Ross *et al.*, 1996; Moran *et al.*, 1996; Bratton *et al.*, 2000) and lead to the formation of DNA adducts in bone marrow cells, liver cells, plasma proteins, and peripheral blood cells (Bodell *et al.*, 1996; Li *et al.*, 1996; Creek *et al.*, 1997; Lindstrom *et al.*, 1999; Troester *et al.*, 2000). The exposure of experimental animals to benzene can result in cytogenic aberrations in the bone marrow and in the peripheral blood and genotoxic effects, which include micronuclei and sister chromatid exchanges in lymphocytes (Tsutsui *et al.*, 1997; Whysner, 2000; Healy, 2001; Yoon *et al.*, 2001).

Recently single cell gel electrophoresis, which is

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called "the comet assay" has been widely used to determine DNA damage including strand breaks, alkali-labile sites, DNA crosslinking, and incomplete excision repair sites after low level exposure to toxicants (Kassie *et al.*, 2000; Meller *et al.*, 2000).

Plappert *et al.* used the single cell gel electrophoresis assay to determine the level of DNA damage in cells from peripheral blood, bone marrow, spleen and liver in benzene exposed mice (Plappert *et al.*, 1994). Mice exposed to benzene as a single oral gavage at 40, 200 or 450 mg/kg resulted in dose-related DNA damage indicated by an increased comet tail length of peripheral blood lymphocytes and bone marrow nucleated cells (Tuo *et al.*, 1996).

Previously we evaluated DNA damage of T-, B-lymphocytes and granulocytes in workers exposed to low level of benzene and found statistically significant effects on B-lymphocytes (Sul *et al.*, 2002).

In the present work, we determined the different level of DNA damage induced in the peripheral T- and B-lymphocytes of rats exposed to low and high levels of benzene. We also found the correlation between DNA damage and the level of trans, trans-muconic acid (*t,t*-MA) in T- and B-lymphocytes. This study provides the first evidence for single strand DNA breaks in T- and B-lymphocytes in rats.

Materials and Methods

Animals

Specific pathogen free male, Sprague-Dawley rats were obtained from the Samtaco Animal Breeding Company (Osan, Korea) and housed in standard laboratory conditions (Tm; 24±2°C, humidity; 50±10% and 12h day and night cycles). Animals were acclimatized to the facility for 1-2 weeks and were observed for abnormal behavior. They had free access to a standard chow diet and drinking water *ad libitum* and were 6-8 weeks old on the first day of exposure.

Experimental design

We used four benzene exposure groups: 10 ppm, 200 ppm, and 400 ppm and an unexposed control for 1, 2, 4, and 6 weeks. The exposure were made 6 h/day, 5 days/week in exposure chamber. To determine *t,t*-MA concentrations, urine was collected after 5 days/weeks exposure. Rats were sacrificed and bloods were collected to determine of DNA damage level.

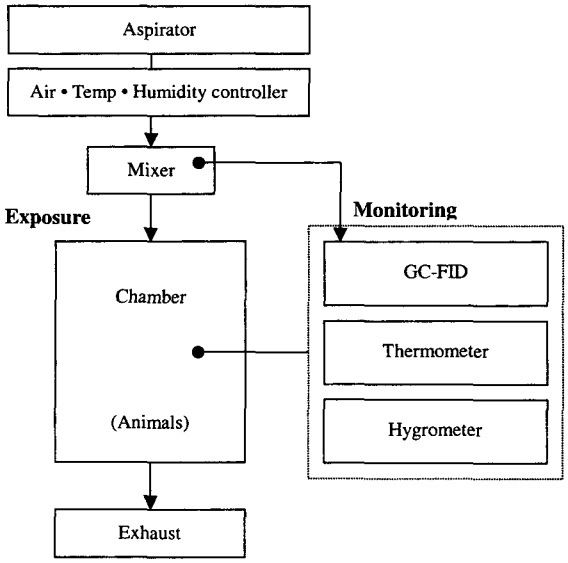

Fig. 1. Schematic diagram of experimental design of benzene exposure.

Benzene Exposure

Benzene exposures were conducted for up to 6 weeks in 1-m^3 stainless steel and glass inhalation chambers in the target benzene concentrations of 10 ppm, 200 ppm, and 400 ppm. Figure 1 shows a schematic of the exposure process. Benzene concentrations were generated by a aspirator (Gastec, Japan) and adjusted by altering the humidified airflow in a second mixing chamber. Exposure chamber air was conditioned to 22±2°C and 50±5% humadity. Benzene concentrations were monitored using a Hewlett Packard Gas Chromatography (Model 6890) fitted with a hydrogen flame ionization detector using helium as a carrier gas through a HP-624 column (30 m × 0.2 mm × 0.33 μm). The actual mean benzene concentration achieved over the six-week study were 10.32±0.54, 194.8±9.84 and 390.4±11.2.

Cell preparation

Blood samples, 3-5 ml of heparinized whole blood, were collected by cardiac puncture from each rat and Comet assay was carried out within 3 hours. T-lymphocytes and B-lymphocytes were positively selected with magnetic beads (Magnetic cell sorting (MACS CD4 or CD45R isolation kit; Miltenyi Biotec) according to the manufacturers instruction. Unfractionated leukocytes were prepared by removal of red blood cells from whole blood by centrifugation with Ficoll-Paque solution. Leukocytes were incubated with each kind of monoclonal

antibody (20 μl of MACS microbeads per 10^7 total cells) for 15 minutes at 6-12°C. After washing cells by adding 10-20X the labeling volume of PBS buffer, the cells were centrifuged at 300×g for 10 minutes, supernatant removed completely and resuspended in appropriate amount of buffer (500 μl of buffer per 10^8 total cells) supernatant removed completely and the cells were applied onto a prepared MS column (Miltenyi Biotec) placed in the magnetic field of a MACS separator and washed with 500 μl buffer. After elimination of negative cells, the column was removed from the MACS separator, placed column on a suitable collection tube and each type of cells was collected and washed with PBS buffer for comet assay.

Comet assay

The comet assay was performed according to Singh with minor modification (Singh *et al.*, 1988). Normal melting point agarose (Ameresco, NMA) and low melting point agarose (Ameresco, LMA) were dissolved in PBS (Gibco BRL) using microwave. In brief, 100 μl of 1% NMA was added onto a fully frosted slides precoated with 50 μl of 1% NMA for a firm attachment and the slides were allowed to solidify with cover slips in the refrigerator for 5 min. After solidification of the gel, the cover slips were removed and lymphocytes in 50 μl mixed with 50 μl of 1% LMA was added. The cover slips were added on the layer and the slides were allowed to solidify in the refrigerator for 5 min. After removing cover slips, 100 μl of 0.5% LMA was added on the third layer and the slides were placed with cover slips again in the refrigerator for 5 min. The slides were submersed in the lysing solution (2.5 M NaCl, 100 mM EDTA-2Na, 10 mM Tris-HCl, pH 10; 1% Triton X-100 and 10% DMSO, pH 10 were added fresh) for 1 hour. The slides were then placed in unwinding buffer (1 mM EDTA and 300 mM NaOH, pH 13) for 20 min and electrophoresis was carried out using the same solution for 20 min at 25 V and 300 mA (0.8 v/cm). After electrophoresis, the slides were neutralized by washing three times with neutralization buffer (400 mM Tris-HCl, pH 7.4) 5 min each and were stained with 50 μl of 10 μg/ml ethidium bromide. The slides were examined using a Komet 4.0 image analysis system (Kinetic Imaging, Liverpool, UK) fitted with an Olympus BX50 fluorescence microscope equipped with an excitation filter of 515-560 nm and a barrier filter 590 nm. For each treatment group, two slides were prepared and each 50 randomly chosen cells (total 100 cells) were scored manually. The Olive tail moments were calculated automatically using the Komet 4.0 image analysis system, Olive tail moment (=(Tail.mean-Head.mean)× Tail%DNA/100), was used for global comet description.

Determination of *t,t*-muconic acid (*t,t*-MA) and creatinine in urine

The high performance liquid chromatographic (HPLC) method of Inoue *et al.* (1989) for *t,t*-MA was carried out with a small modification for urine analysis as follows: Urinary *t,t*-MA was measured by HPLC (Gilson) equipped with Spherisorb ODS 5 mm column (4.6 mm in inner diameter and 300 mm in length). 50-100 ml urine samples were collected and centrifuged immediately at 3,000 rpm for 5 min. The supernatants were purified by solid phase extraction with disposable C18 column and then injected into a HPLC with UV detector. The mobile phase (one volume of methanol mixed with nine volumes of 1% acetic acid) was allowed to flow at a rate of 1.0 ml/min, and the eluates were monitored at wavelength of 259 nm. In order to adjust the levels of *t,t*-MAs in urine samples, urinary creatinine was determined with a Hitachi 747 Computer-Directed Analyzer .

Statistical analysis

Statistical analysis was done by ANOVA for difference of olive tail moments among exposure and control groups, and pearson's correlation between olive tail moments of lymphocytes and urinary *t,t*-MA using SAS/PC 6.12.

Results

DNA damages in T- and B-lymphocytes

The results of the comet assay using rat peripheral T- and B-lymphocytes are shown in Figs. 2 and 3. Figure 2 shows DNA damage in T-lymphocytes. After 1, 2, 4, and 6 weeks of benzene exposure, the mean values of the Olive tail moments of T-lymphocytes of rats exposed to 10 ppm benzene were 1.775±0.173, 1.642±0.198, 2.924±0.615, and 1.739±0.108, respectively ($p<0.05$). The mean value of the Olive tail moment of the control T-lymphocytes was 1.507±0.187. DNA damage of T-lymphocytes in rats exposed for 4 weeks showed the highest value of Olive tail moment and the DNA damage was reduced after 6 weeks of exposures and this was statistically significant. ($p<0.05$). This trend was also observed in rats exposed to 200 and 400 ppm

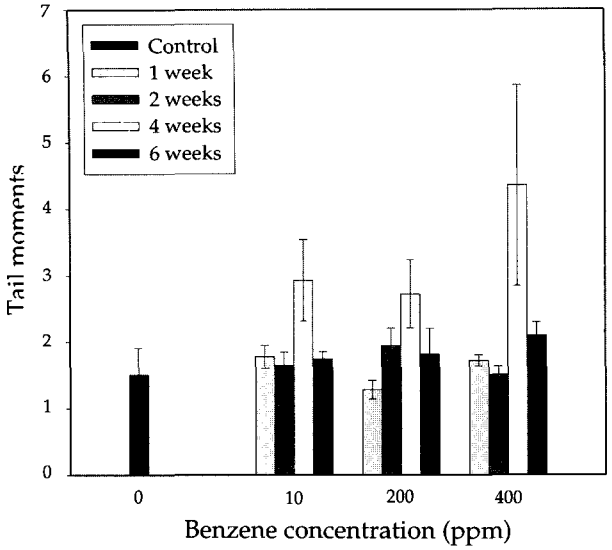

Fig. 2. Distribution of Olive tail moments of T-lymphocytes in benzene exposed rats and unexposed controls. *The values of Olive tail moments at four weeks were the highest encountered all exposure levels by ANOVA(p=0.0004, 0.0007, 0.0002 in 10, 200, 400 ppm respectively).

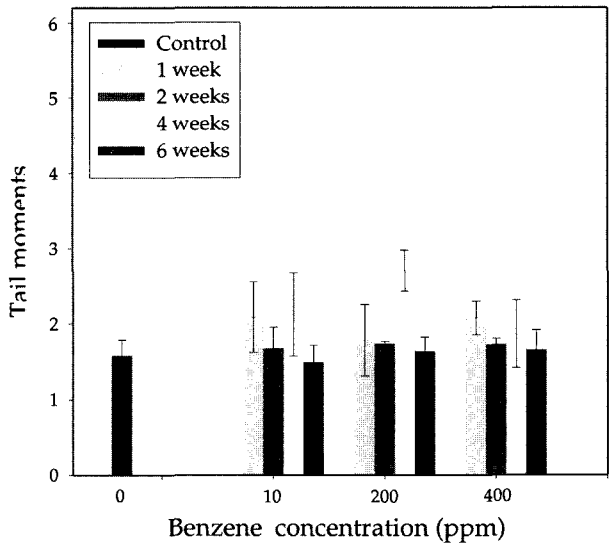

Fig. 3. Distribution of Olive tail moments of B-lymphocytes in benzene exposed rats and unexposed controls. *The value of O ive tail moments at 4 weeks were the highest at a benzene concentration of 200 ppm (p=0.0002).

benzene. After exposure to 1, 2, 4, and 6 weeks of benzene, the mean values of the Olive tail moments of T-lymphocytes in rats exposed to 200 ppm were 1.277 ±0.142, 1.879±0.282, 2.717±0.514, and 1.809±0.388 respectively ($p<0.05$) and for rats exposed to 400 ppm these were 1.713±0.085, 1.494±0.139, 4.351±1.510, and 2.09±0.199 ($p<0.05$).

A significant increase of DNA damages occurred at 4 weeks and this significantly decrease at 6 weeks at all benzene exposure levels (Fig. 2).

After exposure to benzene for 1, 2, 4, and 6 weeks, the mean values of the Olive tail moments of the B-lymphocytes of rats exposed to 10 ppm benzene were 2.091±0.467, 1.671±0.283, 2.099±0.671, and 1.488± 0.226, respectively ($p>0.05$). No significant difference in B-lymphocyte DNA damages was found on increasing benzene exposure times (Fig. 3). The mean value of the Olive tail moment in control B-lymphocytes was 1.579 ±0.206. In control B-lymphocytes and T-lymphocytes, DNA damages were found with no statistical significant difference ($p>0.05$).

The mean values of the Olive tail moments of B-lymphocytes in rats exposed to 200 ppm were 1.783± 0.470, 1.730±0.03, 2.701±0.274, and 1.634±0.185 respectively, after exposure to benzene for 1, 2, 4, and 6 weeks ($p<0.05$) and those of B-lymphocytes in rats exposed to 400 ppm were 2.076±0.223, 1.722±0.077, 1.861±0.450, and 1.652±0.265 ($p>0.05$).

No significant difference in the level of DNA damages in B-lymphocytes was found on increasing exposure time and benzene concentration, except in the case exposure at 200 ppm (Fig. 3).

The correlation between tail moments and *t,t*-MA

The final products of benzene metabolism excreted in urine, *t,t*-MA was used as a biological marker of benzene exposure. *t,t*-MA levels were measured in exposed rats and adjusted by creatinine. The *t,t*-MA levels were increased with increased benzene exposure and became saturated after four weeks of benzene exposure at 200 ppm and 400 ppm. After four weeks, the levels of *t,t*-MA in urine from rats exposed to 10, 200, and 400 ppm fell in the ranged 2.65-2.92, 28.76-30.61, 43.71-46.93 mg/g creatinine. At low levels of exposure to 10 ppm benzene, the *t,t*-MA values remained constantly during the six weeks exposure period (Fig. 4).

The correlation between the levels of *t,t*-MA and the Olive tail moments of T-

lymphocytes was high and statistically significant (r = 0.632, p=0.0001) though a lower correlation was found in the case of B-lymphocytes (r=0.106, p=0.463). According to the exposure levels, correlations in T-lymphocytes were higher and statistically significant except at the 10 ppm level. However, correlations in B-lymphocytes

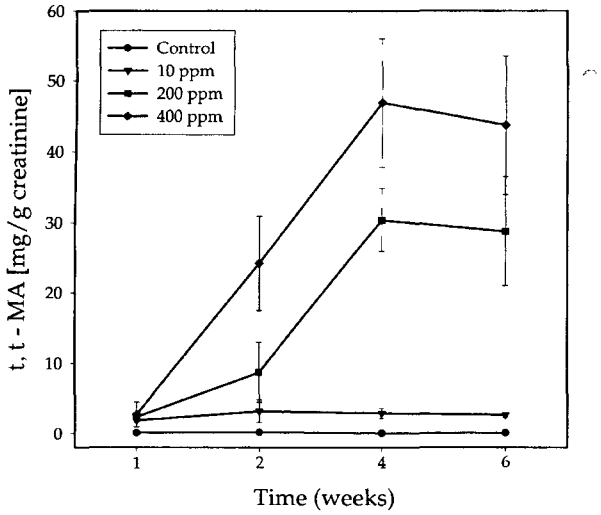

Fig. 4. The *t,t*-MA concentration in urine of benzene exposed rats and unexposed controls.

Table 1. Correlation between Olive tail moments of T- and B-lymphocytes and *t,t*-MA in exposed and control rats

Exposure levels of benzene	T-lymphocytes	B-lymphocytes
0 ppm (N=15)	0.157(p=0.157)	-0.041(p=0.884)
200 ppm (N=15)	0.715(p=0.0028)	0.435 (p=0.106)
400 ppm (N=15)	0.686(p=0.0047)	-0.298 (p=0.282)
Total	0.632(p=0.0001)	0.106 (p=0.463)

were low and statistically were not significant (Table 1).

Discussion

In vivo benzene is converted to benzene oxide, which is rearranged to form other molecules like phenol and premercapturic acid and then is converted finally to the metabolic catechol, hydroquinone, and benzoquinone (Medinsky *et al.*, 1989; Hedli *et al.*, 1997; Hoffmann *et al.*, 1999). These benzene metabolites bind covalently to cellular molecules, proteins and DNA in several tissues and this binding of benzene metabolites to DNA offers the potential mechanism for the inhibition of cell replication or for the initiation of leukemia (Amdur *et al.*, 1992).

On the other hands, nucleophilic xenobiotics such as phenol and hydroquinones are prone to lose an electron and form free radicals in peroxidas catalyzed reaction. These radicals attack sugars, purines and pyrimidines in DNA causing strands breaks (Halliwell and Gutteridge, 1999). Khan *et al.* (1990) proposed that benzene causes the production of hydroxyl radicals and induces apoptosis in lymphocytes. Subrahmanyam *et al.* (1991) hypothesized that benzene induced free radicals contribute to the toxic and leukemogenic effects of benzene. Free radical also generated in yeast by the Salmonella test-negative carcinogen, benzene (Brennan and Schiestl, 1998).

It has been reported that 100 or 200 ppm benzene induces rapid and persistent reductions in femoral B-, splenic T- and B-, and thymic T-lymphocytes in mice lymphocytes (Rozen and Snyder, 1985). However, Robinson *et al.* reported that the absence of immunotoxicological effects upto 200 ppm benzene, in rats exposed for 6 h/day, 5 days/week for 2 or 4 weeks. Only total spleen cell counts were significantly reduced (29%) in rats exposed to 400 ppm after 4 weeks (Robinson *et al.*, 1997).

In human blood cells, the majority of lymphocytes, which are produced by the lymph nodes, spleen, thymus and bone marrow, i.e. T-lymphocytes, which are long lived and have a life-span of from 4 to 10 years. The remaining lymphocytes like B-lymphocytes, representing 15% or so of the lymphocyte population, are short-lived, lasting only 3 to 4 days (Beutler *et al.*, 1995; Brown, 1993).

In a previous study upon benzene workers, we postulated that the comet assay provided evidence that B-lymphocytes are more sensitive to low levels of benzene than T-lymphocytes or granulocytes and this could be ascribed to a greater level of genomic damage in B-lymphocytes than in T-lymphocytes and granulocytes of the primary lymphopoietic organ, the bone marrow (Farris *et al.*, 1997).

However we obtained very different results on DNA damage from the peripheral blood cells of rats exposed to benzene at 10, 200, 400 ppm for 6 weeks. T-cells showed more DNA damage than B-cells, not likely human blood cells. Plappert *et al.* (1994) showed that DNA damage in peripheral blood cells decreased with continued exposure when mice was exposed to 900 ppm of benzene over 4 weeks. They also demonstrated that repair, studied 24 and 48 hours after exposure to 100 and 300 ppm benzene, occurred in peripheral blood cells (Plappert *et al.*, 1994). Tuo *et al.* (1996) postulated that it is possible that the reduction in toxicity observed was underestimated if higher benzene concentrations increase other activation pathway (Tuo *et al.*, 1996).

In a previous study, workers exposed to benzene containing organic solvents on high-speed printing processes, and who did not have a history of an acute high exposure to benzene, but who had been exposed to low levels of benzene from 4 month to 25 years showed

more significant DNA damage in B-lymphocytes and granulocytes than T-lymphocytes (Sul *et al.*, 2002). However, it is believed that an unknown means of reducing DNA damage could have been activated by a high concentration benzene in rats. In addition, rapid replenishment and their short life-span could have given B-lymphocytes a greater repair capacity.

The *t,t*-MA concentration in urine increased with exposure time but became saturated after 4 weeks of exposure, and maintained a constant concentration at 6 weeks. On the other hands, DNA damage revealed was highest in T-lymphocytes but not in B-lymphocytes after 4 weeks of exposure to benzene concentration of 10 200, and 400 ppm. However, DNA damage in blood cells seemed to recover after 6 weeks of benzene exposure in blood cells. Single strand breaks are efficiently repaired by cells with resistance, and this might activate an enzyme repair system after 6 weeks of benzene exposure.

In conclusion, in this study we found that T-lymphocytes contained more single strand DNA breaks than B-lymphocytes in rats exposed to benzene and that this DNA damage was repaired after 6 weeks of exposure to benzene. We postulate that different mechanisms may be incited by long chronic exposure to low levels of benzene, and short acute exposure to high levels, and that an unknown protective mechanism may be activated by high benzene concentrations to reduce DNA damages.

The study to evaluate the repair system activated by exposure to high concentrations and long exposure time to benzene in lymphocytes and other tissues may be important to get more information about the genotoxicity of benzene.

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