Detection of Sperm DNA Damage in Workers Exposed to Benzene by Modified Single Cell Gel Electrophoresis

Bo SONG1, Zhi-ming CAI1, Xin LI2, Li-xia DENG3, Qiao ZHANG3, Lu-kang ZHENG3
1. Dept of Andrology, Peking University Shenzhen Hospital, Shenzhen 518036, China
2. Center of Diseases Prevention and Control of Guangdong, Guangzhou 510300, China
3. Laboratory of Genetic Toxicology, School of Public Health, Sun Yat-Sen University, Guangzhou 510080, China

Objective  To assess the effect of benzene on sperm DNA damage

Methods  Twenty-seven benzene-exposed workers were selected as exposed group and 35 normal sperm donors as control group. Air concentration of benzene series in workshop was determined by gas chromatography. As an internal exposure dose of benzene, the concentration of trans, trans-muconic acid (ttMA) was determined by high performance liquid chromatography. DNA was detected by modified single cell gel electrophoresis (SCGE).

Results  The air concentrations of benzene, toluene and xylene at the workplace were 86.49 ± 2.83 mg/m³, 97.20 ± 3.52 mg/m³ and 97.45 ± 2.10 mg/m³, respectively. Urinary ttMA in exposed group (1.040 ± 0.617 mg/L) was significantly higher than that of control group (0.819 ± 0.157 mg/L). The percentage of head DNA, determined by modified SCGE method, significantly decreased in the exposed group (n=13, 70.18% ± 7.36%) compared with the control (n=16, 90.62% ± 2.94%) (P<0.001).

Conclusion  The modified SCGE method can be used to investigate the damage of sperm DNA. As genotoxin and reprotoxins, benzene had direct effect on the germ cells during the spermatogenesis.

Key words: single cell gel electrophoresis (SCGE); sperm; DNA damage

It was well known that the genotoxicity and the carcinogenic effects of benzene were demonstrated[1,2]. Recently more attention had been paid to the effects of benzene on human
reproduction. These studies focused on the female reproductive system\textsuperscript{[3]} or the semen, such as volume, viscosity and liquefaction\textsuperscript{[4]}. Few articles reported investigating the effects of benzene on sperm DNA that was crucial for human reproduction\textsuperscript{[5]}. The purpose of this study was to assess sperm DNA damage in workers exposed to benzene and to investigate the possible mechanism of benzene in germ cells.

**Materials & Methods**

**Subjects**

Twenty-seven painting workers, exposed to benzene series for over 2 years, were selected as exposed group with mean age of 31.2 ± 5.2 years. Thirty-five normal sperm donors, who had no history of contacting toxic chemicals, were selected as control group with mean age of 34.7 ± 5.9 years. The information about age, length of employment, smoking habit, and diagnostic exposure to radioactive rays was collected by questionnaire. Both the relative data between the exposure and the controls were all matched.

**Reagents**

Normal melting point agarose (NMA), low melting point agarose (LMA), ethidium bromide (EB) and N-lauroylalsarcosinate were purchased from Sigma Chemical Co. Ltd. Tris, proteinase K was from Merck Chemical Co. Ltd. Other chemicals were brought from Guangzhou Chemical Company.

**Air concentration of benzene series in workshop**

Air concentration of benzene series in workshop was determined by gas chromatography. The air samples were collected with a single passive organic vapor monitor (No.GJ-1, Tianjin Labor Institute), which were bore to the cloth at the respiratory zone of workers during the workday.

**Analysis of urine trans, trans-muconic acid**

The urine was collected and stored at −30°C. The concentration of trans, trans-muconic acid (ttMA) was determined by high performance liquid chromatography (HPLC) with coupled columns, and corrected with creatinine.

**Assessment of sperm DNA by modified SCGE**

Semen samples were collected by masturbation after 3-5 d abstinence (13 benzene-exposed workers and 16 normal donors). When liquefied, the semen were stored at −80°C without any chemical preservative.

Frozen semen was thawed at 37°C and washed twice with Ca\textsuperscript{2+} and Mg\textsuperscript{2+} free PBS (pH 7.4), centrifugated at 2 000 r/min, diluted cells with PBS to (4-6)×10\textsuperscript{6}/ml. Cell suspension of 20 µl was mixed with 120 µl LMA (0.65 % in PBS) in a model at 10°C. Eight min later, 2 ml cold lysing buffer (2.5 mol/L NaCl, 0.1 mol/L EDTA, 10 mmol/L Tris, 1% sodium sarcosinate, pH 10, with 1% Triton X-100 and 10% DMSO, mixed just before use for a minimum of 1 h at 10°C) per model was added at 10°C for 1 h.
Lysing buffer was put out, cell mixture was incubated in water incubator (30-40 r/min, 55°C, 3 h) with zymic buffer (2.5 mol/L NaCl, 10 mmol/L Tris, 0.05% sodium sarcosinate, pH 7.4, with proteinase K 0.5 mg/ml, added just before use, 2 ml/model). Then zymic buffer was put out, cell-LMA mixture was washed twice with PBS. Mixture was warmed the drained at 65°C for 2-3 min and cooled to 42°C for use.

Eighty µl NMA (0.65% in PBS at 42°C) was dropped on prepared slides, and covered immediately with coverslide at 10°C for 8 min. Then coverslide was removed, 70 µl cell-LMA mixture was dropped on the top of the first layer, covered with coverslide. Eight min later at 10°C, coverslide was removed.

Slides were placed in electrophoresis tank which was filled with fresh electrophoresis solution (0.3 mol/L CH₃COONa, 0.1 mol/L Tris, pH 10).Slides were left in the buffer for 40 min at 15°C. Electrophoresis condition was 1 h by 13V (0.65 V/cm) and the current was to 130 (130 ± 3) mA by raising or lowing the buffer level. After electrophoresis, Tris buffer (0.4 mol/L Tris, pH 7.4) was added to neutralize the excess akali for thrice and the slides were allowed to sit for 5 min.

Fifty µl EB (20 µg/ml) was added to each slide and covered with coverslide. Slide was placed in a humidified air-tighten container, and analyzed within 48 h. All steps were conducted under dimmed light to prevent the occurrence of additional DNA damage.

Slides were examined under a fluorescence microscope (Zeiss, German, 400×), which was equipped with an excitation filter of 580 nm, and a barrier filter of 590 nm. Fifty cells were scored from each slide. A computer image analysis system (Komet 3.0, Kometic Imaging Ltd.) was used to measure comet parameters. The percentage of head DNA was chosen as used by Hughes, et al[6].

**Statistic**

The concentrations of ttMA measured by geometric means were transformed by natural logarithm. The percentage of head DNA for the sperm violated was measured by the normality test. t- test of SPSS for Windows 10.0 was used.

**Results**

**Air concentrations of benzene series**

The TWA concentrations (time weighted average concentrations) of benzene series at the workshop are showed in Table 1.

<table>
<thead>
<tr>
<th>Benzene series</th>
<th>$\bar{x} \pm s$</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>86.49 ± 2.83</td>
<td>9.14 - 255.80</td>
</tr>
<tr>
<td>Toluene</td>
<td>97.20 ± 3.52</td>
<td>8.98 - 955.84</td>
</tr>
<tr>
<td>Xylene</td>
<td>97.45 ± 2.21</td>
<td>17.14 - 598.71</td>
</tr>
</tbody>
</table>
Concentration of ttMA

The concentration of ttMA was measured by HPLC. After corrected by creatinine, the geometric means concentration of ttMA in test group (1.040 ± 0.617 mg/L, n=27) was significantly higher than that of control group (0.189 ± 0.157 mg/L creatinine, n=37) (P<0.01).

Percentage of sperm head DNA

Percentages of sperm head DNA tested by SCGE (Figure 1) in the two groups are showed in Table 2. The percentage of exposed groups significantly decreased compared with the control (Table 2, P<0.01).

Table 2  Percentage of head DNA tested by SCGE (X ± s)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Percentage of head DNA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exposed</td>
<td>13</td>
<td>70.18 ± 7.36</td>
</tr>
<tr>
<td>Control</td>
<td>16</td>
<td>90.62 ± 2.94</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Discussion

In recent years, more attention has been paid to the genetic damage in germ cells induced by environmental chemicals. However, there was difficulty in the method to measure the DNA damage. In the mature process of spermateliosis, the histone connected with DNA was replaced by protamine that was rich of -S-S- bands. Hence more compact superhelix structure was formed. The tight condensation of sperm chromatin made it difficult to separate DNA with protamine. The key step of the sperm SCGE was to separate DNA strand from protamine. Kinds of methods had been used to solve this problem, such as the use of lithium diiodosalicylate[8] and RNase[9]. However, those methods were not all necessary, just as there was few RNA in nuage. In the modified SCGE, we changed the process by treating cells first and making the gel later. Based on the characteristic of proteinase K, enzyme reaction condition was adjusted from 37°C, 15 h[8] or 22 h[7,9,11] to 55°C, 3 h.
These changes shortened the experimental period and made it easy to control the process and avoided gel unstuck.

Benzene had been widely used in industry and existed not only in the air of factory but cigarette\(^2,3\). As mutative and reproductive toxin, it was reported that semen quality, including the volume, viscosity and liquefaction, decreased in benzene-exposed males\(^5\). However there were few studies on assessing the effect of benzene on DNA and its mechanism was not clear\(^13\). In this paper, we detected sperm DNA damage in benzene-exposed workers.

As occupational investigation showed, the air concentration of benzene in workplace (86 mg/m\(^3\), 26 ppm) was 2 fold higher than that of China national allowance concentration (40 mg/m\(^3\)), or 55 fold higher than that of ACGIH (American Conference of Governmental Industrial Hygienists), while those of toluene and xylene were not beyond. As an biomarker of benzene, the exposure concentration of ttMA was significantly higher than that of the controls. From the SCGE results, we known that the percentage of sperm head DNA (70.2\%) in exposed group was significantly less than that of the controls (90.6\%). It suggested that sperm DNA damage was induced in workers exposed to higher concentration of benzene. Benzene could induce DNA breakage in germ cells.

Because the mechanism was not clear, it was believed the reactive metabolite of benzene but benzene affected cells\(^13\). One of the underlying mechanisms was believed to be the oxidative damage caused by its metabolites\(^14,15\). A good correlation-ship was found between 8-hydroxyguanosine and the groups exposed to external and internal benzene existed\(^15\). As a lipophilic substance, benzene was easy to pass the blood testicular barrier and diffused to the testicular epithelial cells. Benzene could be metabolized to active metabolites by the profuse enzyme in the epithelial cells. These metabolites might be the direct factors that affected the spermatogenesis, caused the sperm DNA damage, and led to the breakage of DNA.

**References**


(Received on May 20, 2005)