XPD expression and its effect on methylation on Benzene-induced blood toxicity

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Abstract: Benzene is a known carcinogen that mostly targets and affects the hematopoietic system. It may cause a reduction of whole blood cells, aplastic anemia or even leukemia. To understand the molecular mechanisms of benzene-induced blood toxicity, the epigenetic changes of a deoxyribonucleic acid (DNA) repair gene, Xeroderma pigmentosum complementation group D (XPD), were investigated. Sprague-Dawley (SD) rats and K562 cells were exposed to different concentrations of benzene and its metabolite, hydroquinone. The results showed that both compounds may lead to DNA damage in a dose-dependent manner. Moreover, when their exposure reached a certain dose and time, the expression of XPD messenger ribonucleic acid (mRNA) and protein level increased. Measurement of the methylation status of the XPD gene promoter region in exposed cells revealed that the methylation rate of the XPD gene among groups did not differ significantly. The results of the present study suggested that the changes in XPD mRNA expression and protein level induced by exposure to benzene and its metabolite hydroquinone were not attributed to the abnormal methylation of the XPD gene.

Keywords: Benzene; Hydroquinone; Xeroderma pigmentosum complementation group D; methylation

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1. Introduction

Benzene is a recognized risk factor for leukemia in occupational tumors in China[1-3]. Long-term exposure to low concentrations of benzene can cause a reduction of blood cells, bone marrow hyperplasia, the occurrence of leukemia in severe cases and other hematopoietic system damage[4-6]. With the increased industrialization in recent years, many non-occupational groups have been exposed to low concentrations of benzene due to interior renovation, and vehicle emissions among other reasons in daily life[7, 8]. It was therefore important to investigate the mechanism behind the toxicity of benzene in blood[9]. At present, studies suggest that benzene is an indirect carcinogen and its metabolite, hydroquinone (HQ), causes poisoning in the bone marrow[10, 11]. HQ directly attacks DNA molecules by oxidizing or combining with DNA to form adducts, ultimately leading to the breakage of DNA strands, gene mutations and chromosomal aberrations[12].

Organisms have inherent complete repair mechanisms for DNA damage. Nucleotide excision repair (NER) is one important repair pathway for human DNA damage in which Xeroderma pigmentosum complementation group D (XPD) plays a vital role in the NER[13]. XPD protein is a DNA-unwinding enzyme located near the site of DNA damage[14]. Unwinding the damaged DNA double-strand forms one of the three rate-limiting steps in NER[15]. XPD gene mutation and its abnormal expression are related to the occurrence of several tumors such as breast cancer, thyroid cancer and prostate cancer [16-18], indicating that the abnormal XPD gene has an important role in the occurrence and development of tumors.

Recent studies have shown that epigenetic modification is important in the understanding of the toxic effects of compounds. DNA methylation is one of the most important epigenetic modifications. Various toxic chemical compounds such as arsenic, nickel, and chromium can cause methylation pattern disorder and histone modification changes, thus affecting gene expression [19-21]. Currently, studies on the effect of benzene on the methylation model of damaged DNA repair gene XPD have not been able to explain the role of the XPD gene in the process of benzene blood toxicity. Further investigations are therefore needed.

Treatment of Sprague-Dawley (SD) rats and K562 cells using benzene and its metabolite HQ was used to investigate the role of XPD gene expression and its methylation in DNA damage. The effect of benzene on XPD mRNA transcription and protein expression was also analyzed.

2. Materials and Methods

2.1. K562 cell culture and exposure

Human leukemia cell line, K562 (Tissue Engineering and Stem Cell Experimental Center, Guizhou Medical University) was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Beijing Sijiqing Company, China), 100 U/mL penicillin and 100 U/mL streptomycin at 37°C in 5% CO2 under saturated humidity. The cell culture medium was changed daily or every two days and cell splitting was done once. Cells at a logarithmic growth phase and with cell viability of more than 98% were seeded in 75 ml flasks at the density of 2*105 cells/ml. After HQ (Sigma, USA) was exposed to cells at final concentrations of 0 μmol/L, 15 μmol/L, 30 μmol/L, and 60μmol/L for 24 h respectively, the cell suspension was collected in 15 ml centrifuge tubes followed by centrifugation at 1000 rpm for 5 mins. After the removal of the supernatant, the remaining cell pellet was washed twice with phosphate-buffered saline (PBS) and the same amount of fresh medium was added for further culturing. The following day, HQ treatment was repeated. Three additional HQ treatments were performed as per HQ repeated interval exposure of 72 h method[22]. After the final exposure, cells were collected.

2.2. SD rat exposure and preparation of bone marrow nucleated cell suspension

The present study was approved by the Animal Ethics Committee of Guizhou Medical University. After a week of acclimatization, 30 healthy male SD rats (Chongqing Animal Center, China) were randomly assigned to 5 groups (n=6): blank control, solvent control, benzene low-dose (200 mg/kg), benzene medium-dose (400 mg/kg), and benzene high-dose (800 mg/kg)[23]. Benzene (Tianjin Ruijinte Company, China) was dissolved in corn oil and the blank control group did not receive any treatment. The rats in the exposure groups and solvent control group were gavaged once daily for 28 days. Twenty-four hours after the last exposure, SD rats were anesthesia and euthanized via cervical dislocation. Both sides of the metaphyseal were cut to take out the femur and bone marrow cells which were washed out with 10 ml PBS before filtering once by size number 5 needles. The solution was then centrifuged at 1000 rpm for 5 mins and the supernatant was discarded. After the addition of 1.5 ml red cell lysate, the solution had a whirlpool oscillation for 15s and was then ice bathed for 15 min to lyse red blood cells in the bone marrow completely. The cell lysate was then centrifuged at 1000 rpm for 5 mins followed by the removal of supernatant and washing with cold PBS was done once. Bone marrow cell suspension was made by blowing mix.

2.3. Changes of peripheral blood in SD rats by whole blood cell analyzer

After the exposure to benzene, rats were sacrificed at the femoral artery and 2 ml blood was collected in ethylenediaminetetraacetic acid (EDTA) anticoagulant tubes. After mixing the blood with upside-down movements, automatic whole blood cell analyzer was used to measure routine blood WBC, RBC, Hb, PLT and other indicators.

2.4. Changes of femoral hematopoietic tissue in SD rats by histological analysis

One side of the complete femur was collected after rats were culled and then fixed with 10% paraformaldehyde for 3 days. Decalcification was then performed d for a week. The femur was then processed as follows: conventional dehydrating, transparent, dipping in wax, embedding, slicing, HE staining, sealing for microscopic examination, and observing hematopoietic histopathological changes.

2.5. DNA damage detected by single-cell gel electrophoresis (SCGE)

90 μl of 1% ordinary melting point agarose (pre-heated at 45°C) was spread onto a matte slide to form a first layer of gel. The collected cell suspension was adjusted to a concentration of 1*106 cells/mL and 40 μl of cell suspension was added to 360 μl of 0.6% low melting point agarose (37°C). A 100 μl of the mixture was then added to the first layer of gel before covering the coverslip to make it evenly distributed. The freezing process to form the second layer of gel was then done at 4°C for 10 mins. The slide was refrigerated in the cell digestion solution for 1 h and was then placed near the anode side of the horizontal electrophoresis tank. Freshly prepared alkaline electrophoresis buffer was added until the slide was fully covered. After unwinding in darkness for 20 mins, electrophoresis was performed at 24V, 300 mA for 20 mins before the slide was taken out and washed three times with 0.2 mol/L Tris-HCL buffer f. After drying, the slide was stained with 50 μL of 20 mg/L EB, covered with a coverslip, and then placed in the wet box to avoid light exposure. The cells were observed under a positive fluorescence microscope and pictures of 10 visions were randomly taken under the 200x objective lens. Fifty cells were analyzed. Comet Score software was used to analyze the results of cell images and DNA damage level was measured using Olive tail moment (OTM) (Formula 1).

OTM = (Tail mean–Head mean)*percentage of DNA in the tail (Formula 1)

2.6. Transcription level of XPD mRNA detected by Real-time fluorescence quantitative PCR (QPCR)

Total RNA was extracted from the Triazol reagent as per protocol and was dissolved in 25 μl of DEPC-treated ddH2O before measuring its concentration and purity using a UV spectrophotometer. 1 μg RNA with a ratio of RNA 260nm/280nm ratio of 1.8 to 2.0 was reverse transcribed into cDNA. The Taqman probe was used to quantify the PCR reaction with cDNA as a template. Primers and probes were designed and synthesized by the Dalian TaKaRa Company. Sequences are shown in Table 1. The 20 μl reaction system was: 14.4 μl DEPC-treated ddH2O, 2 μl 10x PCR buffer, 1 μl MgCl2 (50mM), 0.5 μl dNTPs (10 mM), 0.3 μl upstream and 0.3 μl downstream primers (10 μM), 0.3 μl Taqman probe (10 μM), 0.2 μl Taq enzyme (0.025U/μl), and 1.0 μl template. Three replicates were performed for each sample, and instead of cDNA, 2 μl RNase-free deionized water was used as blank control. Reaction conditions were: 95 °C for 2 min; 95°C for 10 s, and 60°C for 30 s, a total of 40 cycles.

cDNA was diluted 10 times to prepare a dilution standard which was used as a template for QPCR. The cycle threshold (CT) was set as the x-axis and the logarithm of DNA concentration was in the y-axis to plot the standard curve. The actual amplification efficiency (E) and correlation coefficient (r) of each target gene were obtained. According to the E, β-actin was the internal reference gene and the relative expression of each gene was calculated by the Pfaffl Method (Formula 2)[24]. (E target gene and E internal reference gene represent the E of the target gene and the internal reference gene respectively).

Relative expression of target gene = $(1+E_{\text{target gene}})^{\Delta Ct\ \text{target gene (sample-control)}} / (1+E_{\text{internal reference gene}})^{\Delta Ct\ \text{internal reference gene (sample-control)}}$ (Formula 2)

Table 1. Taqman probe for fluorescence quantitative PCR primers and probe.

Source	Gene Name	Primer Name	Sequence (5′- 3′)
K562 cells	XPD	D-F	GTACGACTACATCTACCC
		D-R	CACAGTTCTTGAGCAGTA
		D-P	(FAM) TTGGTCACCTCCAGCGGATAT (BHQ1)
	β-action	B-F	GACTTAGTTGCGTTACACCCTTTC
		B-R	GCTGTCACCTTCACCGTTCC
		B-P	(FAM)TGACAAAACCTAACTTGCGCAGAAAACA(BHQ1)
SD rats	XPD	D-F	CATTCTGCACTTCAGCTGTATGG
		D-R	AGACTGGAAGCGTTCAAACACA
		D-P	(FAM)CGCCTCCTTGGCCATCAAGC(TAMRA)
	β-action	B-F	CTGGCCTCACTGTCCACCTT
		B-R	GGGCCGGACTCATCGTACT
		B-P	(FAM)CAGCAGATGTGGATCAGCAAGCAG(TAMRA)

2.7. Expression level of XPD protein by immunoblotting (Western blotting, WB)

The nuclear protein was extracted using the Nuclear protein extraction kit (Jiangsu Beyotime Biotechnology Research Institute, China) and was quantified using Bicinchoninic acid (BCA) protein quantification kit (Jiangsu Beyotime Biotechnology Research Institute, China). The protein was aliquoted and stored at -80°C. 20 μg of nuclear protein was used for polyacrylamide gel electrophoresis and was then transferred to PVDF membrane. Non-specific binding was blocked using 5% skim milk at room temperature for 2 h. The membranes were then incubated in appropriate mouse anti-human XPD monoclonal antibody (1:600, Abcam, USA) and rabbit anti-mouse XPD monoclonal antibody (1:7000, Abcam, USA) overnight at 4°C. The following day, after the membranes were washed, they were incubated with appropriate secondary antibody. The protein was detected using enhanced chemiluminescent agent (ECL) and β-actin was as internal reference. Image J software was used to scan the gray scale of the X-ray film and to analyze the relative expression of XPD protein.

2.8. Methylation status of XPD gene by bisulfite sequencing (BSP)

2.8.1. Prediction of XPD gene CpG island and design of methylation detection primer

The XPD (NG_007067) gene sequence was obtained from GeneBank, which was then combined with the Ensembl database to analyze the sequence of the XPD gene promoter region. Methyl Primer Express v1.0 and MethPrimer online software (http://www.urogene.org/methprimer/index1.html) were applied to predict the CpG island in the XPD gene promoter region. The criteria of CpG islands (Gardiner-Garden and Frommer, 1987): GC content was more than 50%, the ratio of observed and expected value in CpG dinucleotide reached 0.6, and the length of DNA sequence was at least 200 bp. Both software predictions showed that the XPD gene had a CpG island. The predicted CpG island is shown in Figure 1.

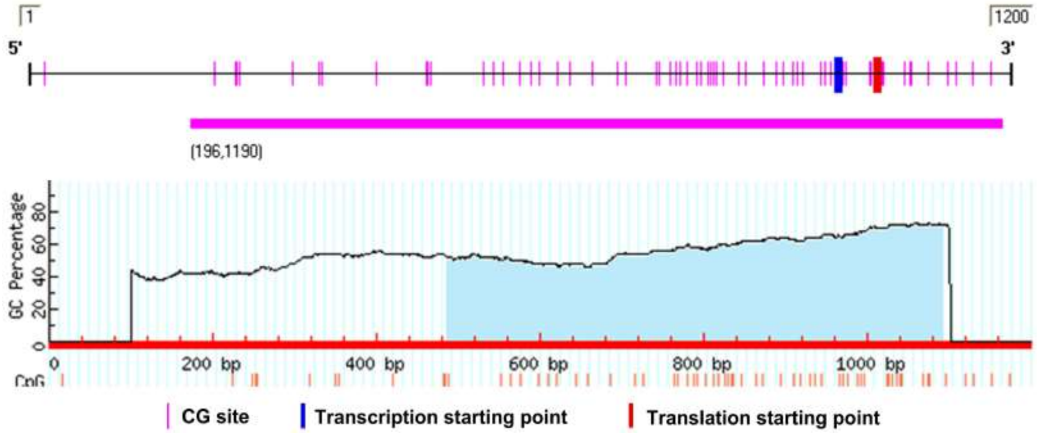


Figure 1. Predicted CpG Island in XPD Gene Promoter Region.

2.8.2. Measurement of methylation status of XPD gene promoter region

Cell genomic DNA was extracted using DNA extraction kit. Its concentration and purity were detected using UV spectrophotometer. An amount of 0.5 μg DNA with a DNA260nm/280nm ratio of 1.7 to 2.0 was used for bisulfite modification following instructions in the EZ DNA Methylation-Gold Kit (ZYMO RESEARCH, USA). The bisulfite-modified DNA was used as a template and amplified with BSP primer. Primer sequences are shown in Table 2. The 25 μl reaction system was as follows: 12.5 μl buffer, 4 μl dNTPs (2.5 mM), 0.5 μl upstream and 0.5 μl downstream primers (10 μM), 1 μl bisulfite-modified DNA, 0.2 μl DNA polymerase, and sterilized ddH2O. Reaction conditions were as follows: pre-denaturation at 95°C for 3 mins, 95℃ for 30 s, 55°C for 30 s, 72°C for 1 min, a total of 35 cycles, and the last extension was at 72°C for 5 mins. The product was electrophoresed by 1.5% agarose gel and was recycled and purified by Axygen gel recycle kit (Dalian TaKaRa Company, China). The purified product was subjected to TA cloning and was then transformed into competent bacteria. A 100 l of the bacterial solution was evenly applied to ampicillin-resistant LB plate and cultured at 37°C until monoclonal colonies were formed. 10 clones were selected from the plate and were sent to Shanghai Invitrogen Company for sequencing.

Table 2. BSP Amplification Primer.

Source	Name	Sequence (5’→3′)	Fragment size
K562 cells	XPD-F	GGAGGATTAAATTTTAGTGAATGAGA	328bp
	XPD-R	ACCCCCTTCTCACTTCATAAC	
SD rats	XPD-F	AGGAAGGTGGTGTTTTAGGTT	328bp
	XPD-R	AAAATAAACCAACAACCCATC	

2.9. Statistical analysis

SPSS 17.0 statistical software was used to analyze the experimental data. Measurement data was expressed as ±s. Methylation data was analyzed using the chi-square test. One-way ANOVA was used for blood factor, DNA damage, XPD gene mRNA and protein expression data analysis. LSD test was used for further comparison among two groups. Differences with $p<0.05$ were considered to be statistically significant

3. Results

3.1. The reduction of peripheral red and white blood cells in rats caused by benzene

As seen from Table 3, there were no significant differences in WBC, RBC, Hb and PLT between the control group and the solvent group ($P>0.05$), indicating that the benzene solvent corn oil did not affect the blood system in rats. WBC levels in the three benzene-exposed groups, HBC levels in the high-dose group and Hb levels in medium- and high-dose groups were significantly lower ($P<0.05$) than those in the control group while, PLT levels in the three benzene-exposed groups did not record statistically significant differences ($P>0.05$) from those in the control group. These results indicate that benzene may have a certain toxic effect on peripheral blood cells, and may reduce the number of both red and white cells, but benzene may not have an obvious impact on platelets.

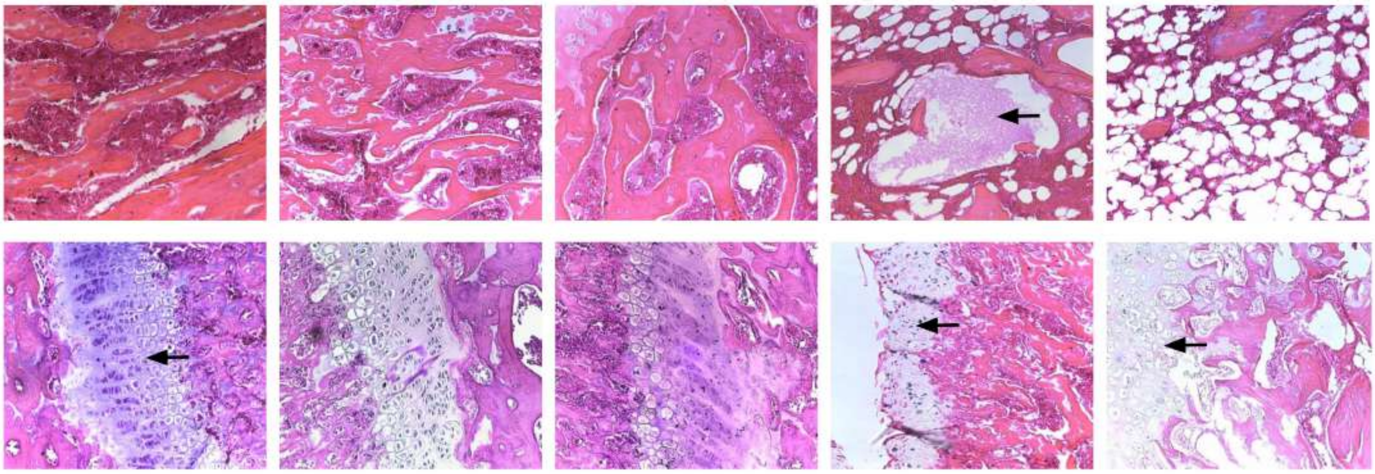
Table 3. Results of the peripheral hematological indexes in SD rats (X±s, n=6).

Group	WBC (109/L)	RBC (1012/L)	Hb(g/L)	PLT(109/L)
Blank control group	8.76±1.60	9.05±0.37	165.50±6.83	933.67±19.86
Solvent control group	8.36±1.41	8.52±0.65	160.50±7.00	1023.00±21.32
Low-dose group	5.85±0.78^{ab}	8.96±0.23	163.83±5.64	1011.67±17.13
Med-dose group	5.77±1.37^{ab}	8.49±0.34	154.83±5.60^{abc}	981.17±12.62
High-dose group	5.49±1.17^{ab}	8.37±0.15^{a}	151.83±2.93^{abc}	885.17±15.46

Note: ^{a} vs blank control group, $P<0.05$; ^{b} vs solvent control group, $P<0.05$; ^{c} vs benzene low-dose group, $P<0.05$.

3.2. Benzene can destroy the hematopoietic tissue in rats

The HE staining results of bone marrow hematopoietic tissues for each group are shown in Figure 2. There were plenty of hematopoietic cells with close arrangement and clear trabecular bone structure and more bone marrow nucleated cells in the blank control group. In the exposed groups, with the increased dose, the trabecular bone had a more obvious fracture, and the chondrocyte layer was disordered with ruptured cells. In addition, hematopoietic cells were reduced and fat droplets increased. These results indicate that benzene may lead to the damage of bone marrow hematopoietic tissues in rats. The higher exposure dose resulted in serious damage of the same.

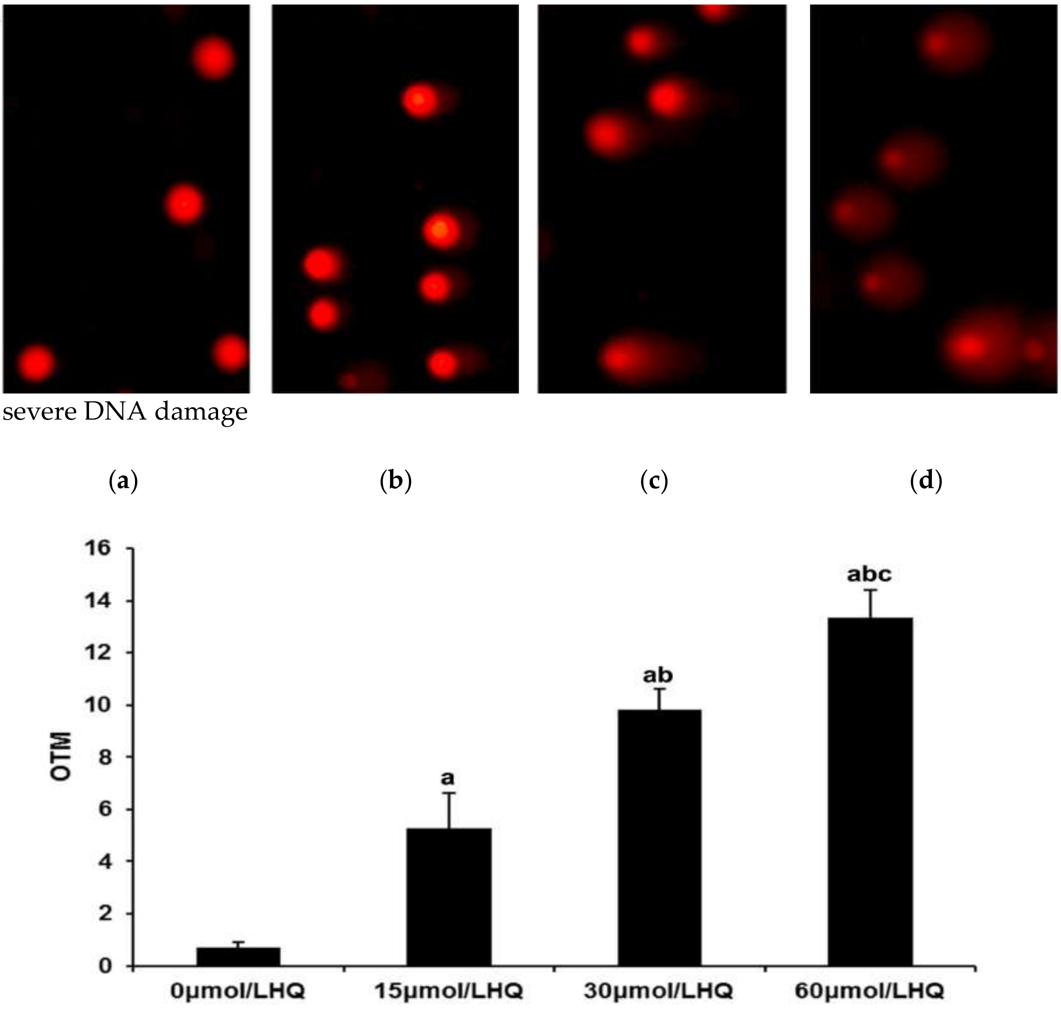


(**a**) (**b**) (**c**) (**d**) (**e**)

Figure 2. HE staining results of bone marrow tissue in different treatment groups. (**a**) Blank control group; (**b**) Solvent control group; (**c**) Low-dose group; (**d**) Medium-dose group; (**e**) High-dose group.

3.3. Benzene and HQ can cause cell DNA damage

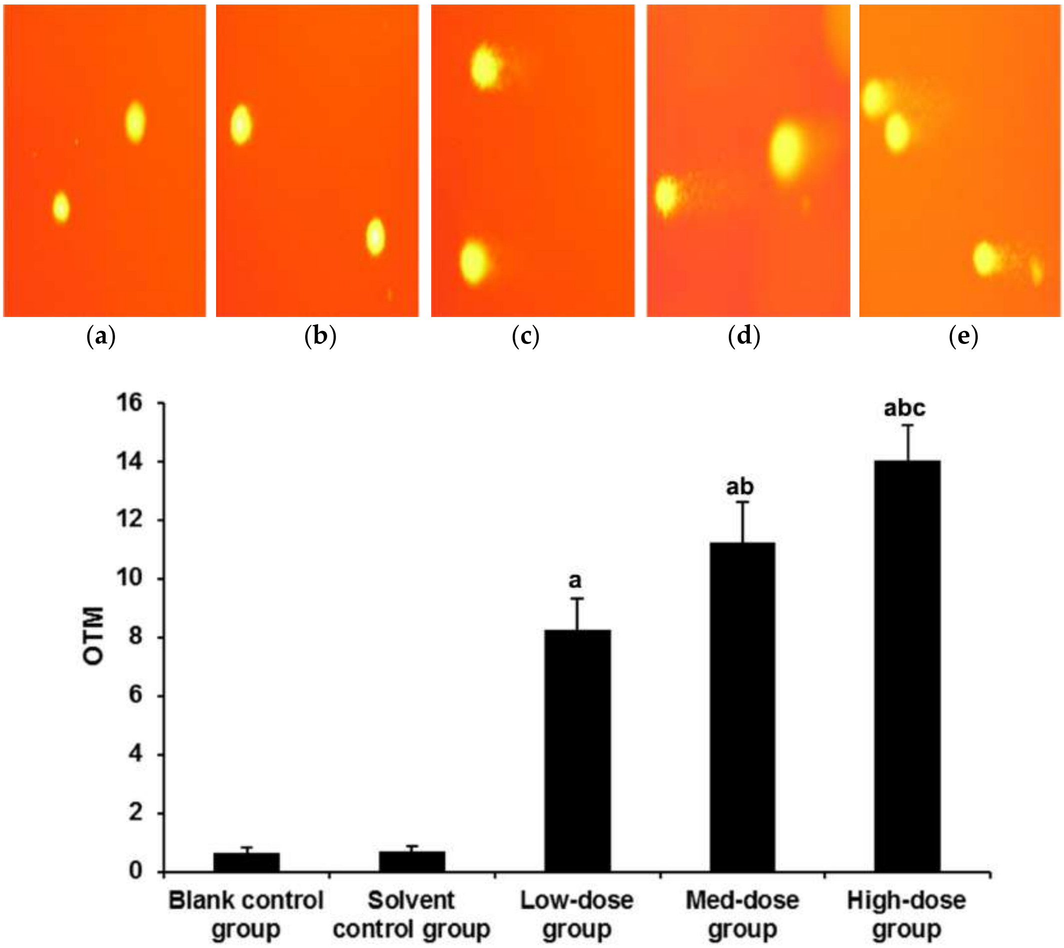
Single-cell gel electrophoresis results are shown in Figure 3 and Figure 4. In the blank control group, most cells were round and lacked tailing while the exposed cells had more obvious comet-like changes in the shape of a small head and a big fan-shaped tail, which were broken small molecule DNA fragments. The OTM obtained from the image acquisition was used to reflect the DNA damage in cells. The increment in OTM reflected the severity of DNA damage. The exposure of K562 cells to HQ resulted in a significant increase in OTM when compared with the blank control group (P<0.05). There was no significant difference in OTM between blank and solvent control groups after the exposure of benzene to SD rats (P>0.05). Compared with the control group, OTM increased significantly in three benzene exposure groups (P<0.05), indicating that benzene and HQ may cause DNA damage in cells. The higher exposure dose resulted in severe DNA damage



(**a**) (**b**) (**c**) (**d**)

(e)

Figure 3. Effect of HQ on DNA damage in K562 Cells. (**a**) 0 μmol/L HQ treatment group; (**b**) 15 μmol/L HQ treatment group; (**c**) 30 μmol/L HQ treatment group; (**d**) 60 μmol/LHQ treatment group; (e) DNA damage after HQ exposure in K562 cells. a vs control group, P<0.05; b vs 15μmol/LHQ, P<0.05; c vs 30μmol/LHQ, P<0.05.



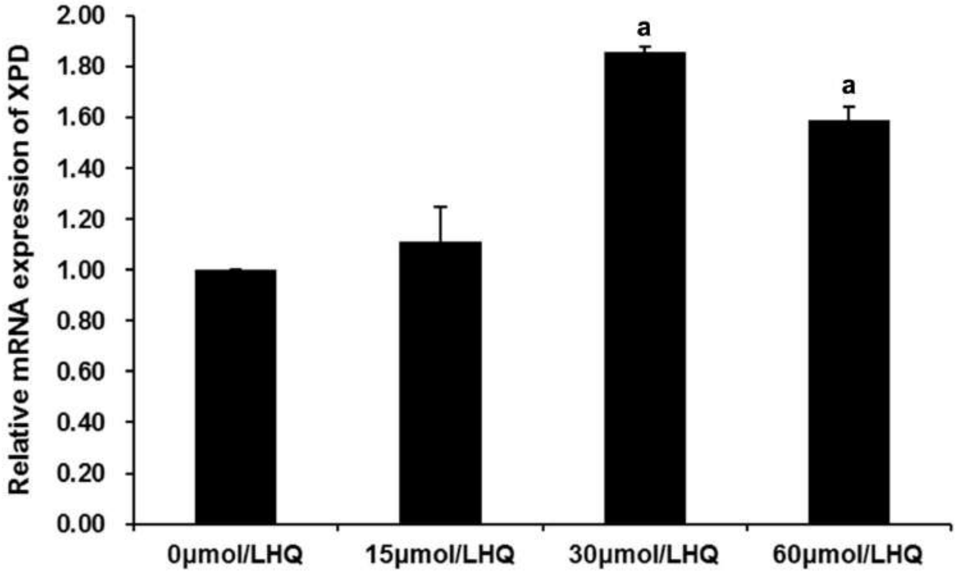
(**a**) (**b**) (**c**) (**d**) (**e**)

(**f**)

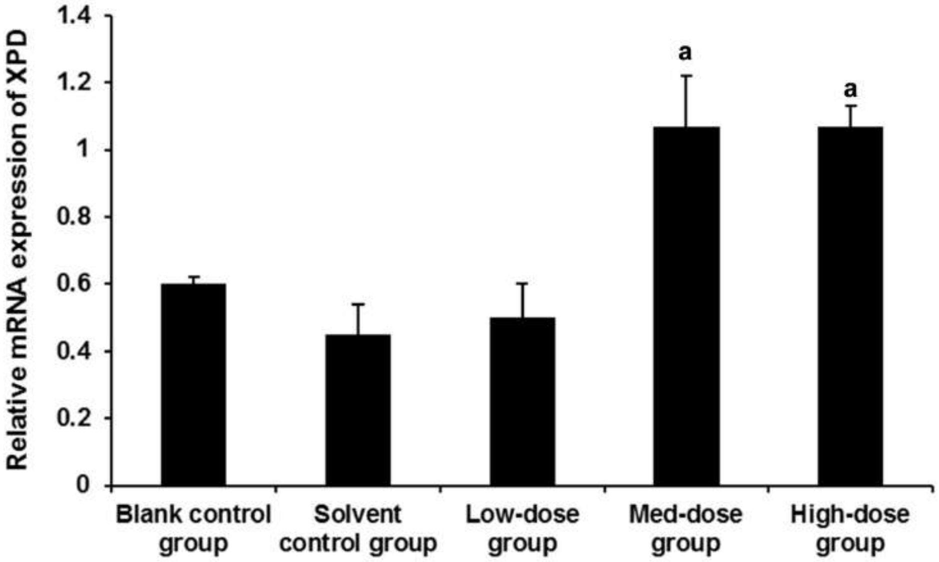
Figure 4. Taqman probe for fluorescence quantitative PCR primers and probe sequences. (**a**) Blank control group; (**b**) Solvent control group; (**c**) Low-dose group; (**d**) Medium-dose group; (**e**) High-dose group; (f) DNA damage after benzene exposure in bone marrow cells of SD rats. a vs control group; b vs low-dose group.

3.4. Upregulation of XPD mRNA expression

The relative expression of each target gene was calculated according to Formula 2. There was no significant difference in XDP mRNA expression between the 15 μmol/L exposed group and control group (P>0.05) while its expression in 30 μmol/L and 60 μmol/L exposure groups significantly increased compared to the control group (P<0.05) (Figure 5a). There was no significant difference between the blank control group and the solvent control group after benzene exposure to SD rats (P>0.05). The mRNA expression between the low-dose group and the control group did not show a significant difference (P>0.05). The expression of mRNA in the medium- and high-dose groups was significantly higher (P<0.05) than that in the control group (Figure 5b).



(**a**)

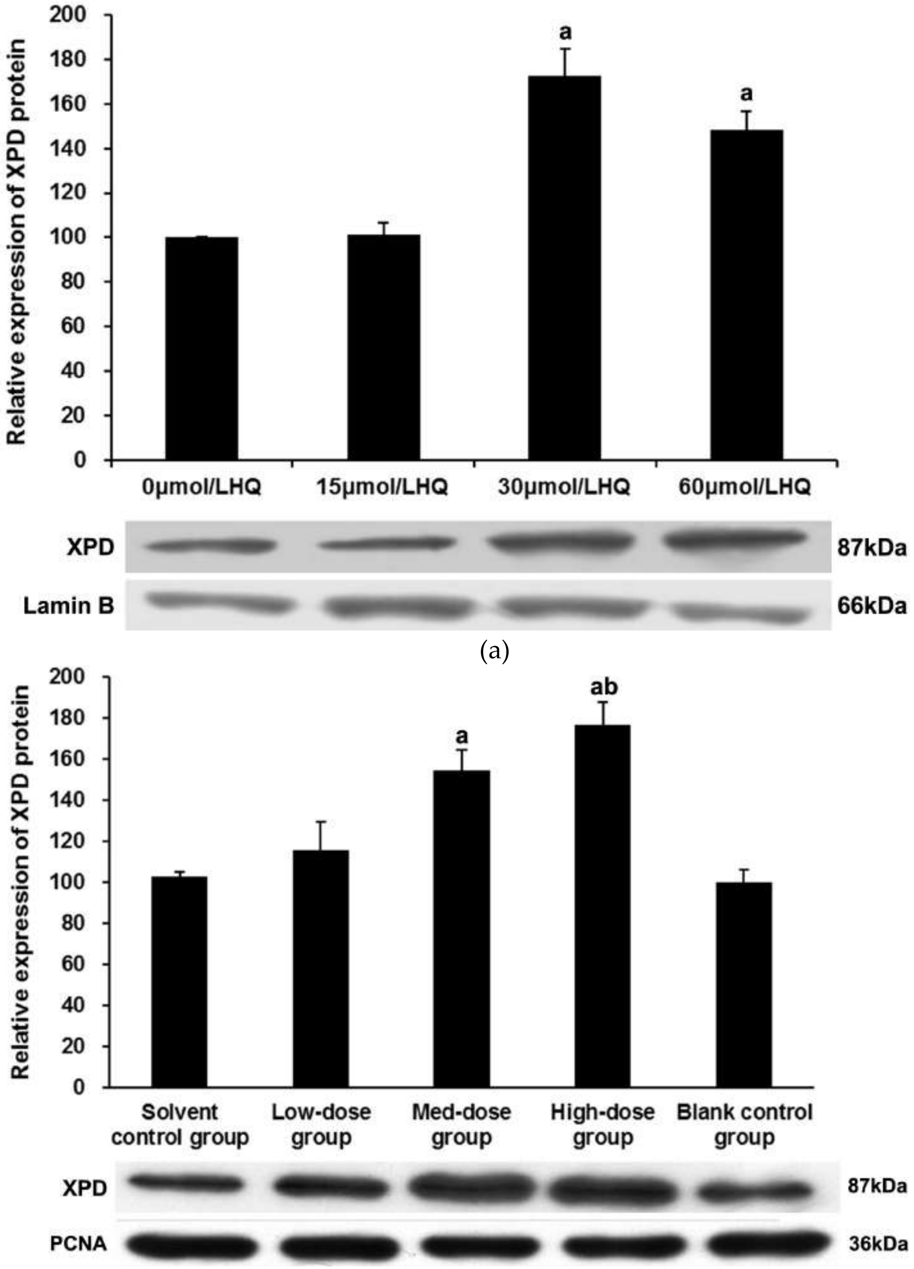


(**b**)

Figure 5. Expression of XPD mRNA. (**a**) Expression of XPD mRNA in HQ-exposed K562 cells; (**b**) Expression of XPD mRNA in benzene-exposed SD rat bone marrow cells. a vs control group, $P<0.05$.

3.6. Upregulation of XPD protein expression

After HQ exposure to K562 cells, there was no significant difference in the relative expression of XPD protein in 15 μmol/L HQ group compared with the control group ($P>0.05$) while the expression of XDP protein increased significantly in the 30 μmol/L and 60 μmol/L HQ groups ($P<0.05$) (Figure 6a). After benzene exposure to SD rats, there was no significant difference between blank control group and solvent control group ($P>0.05$) in the XPD protein expression level. The expression of XPD protein in all the exposure groups was significantly higher ($P<0.05$) than in the control group and there statistically significant differences between any two exposure groups ($P<0.05$) (Figure 6b).



(a)

(b)

Figure 6. Expression of XPD protein. (**a**) Expression of XPD protein in HQ exposed K562 cells. a vs 0μmol/LHQ, P<0.05; (**b**) Expression of XPD protein in benzene-exposed SD rat bone marrow cells. a vs control group P<0.05; b vs medium-dose group.

3.5. The methylation status of the XPD gene did not change

The results of methylation positive control sequencing showed that all the CG dinucleotides were retained in the amplified region and the cytosine (C) of the non-CG locus were all transformed into thymine (T). The conversion rate of the non-CG locus cytosine reached 100%, indicating that the bisulfite had been modified completely to meet the experimental requirements (Figure 7).

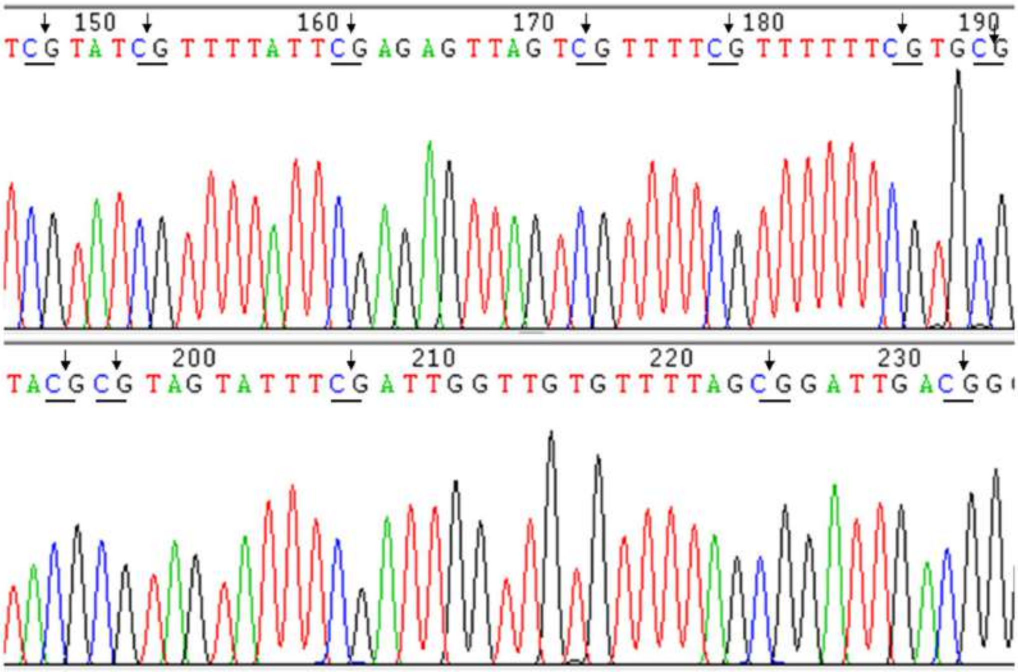
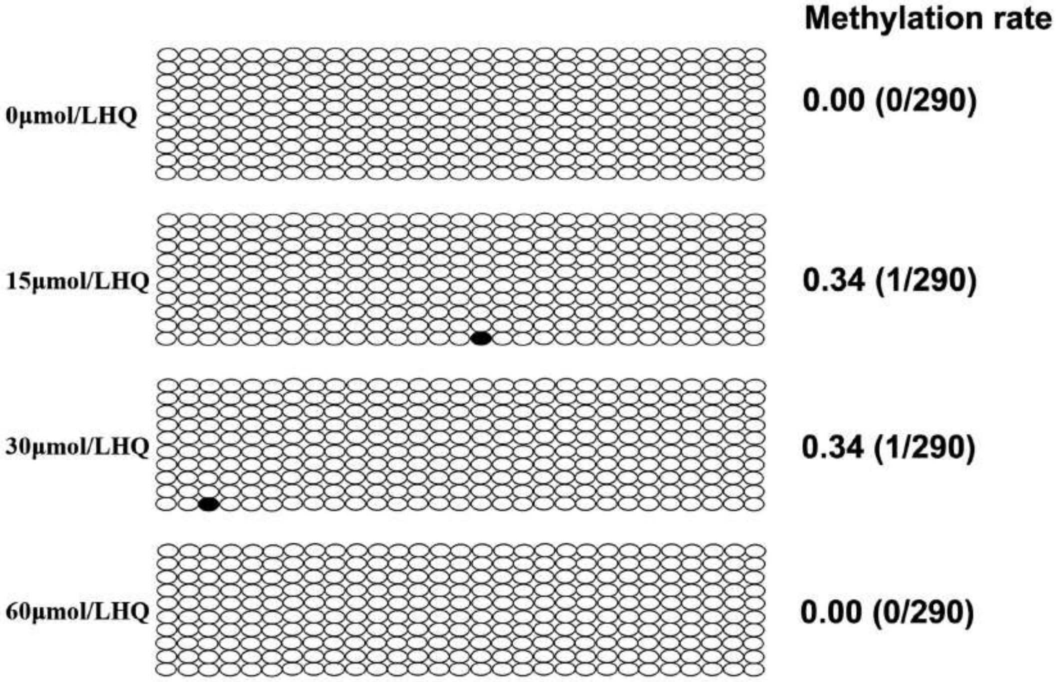
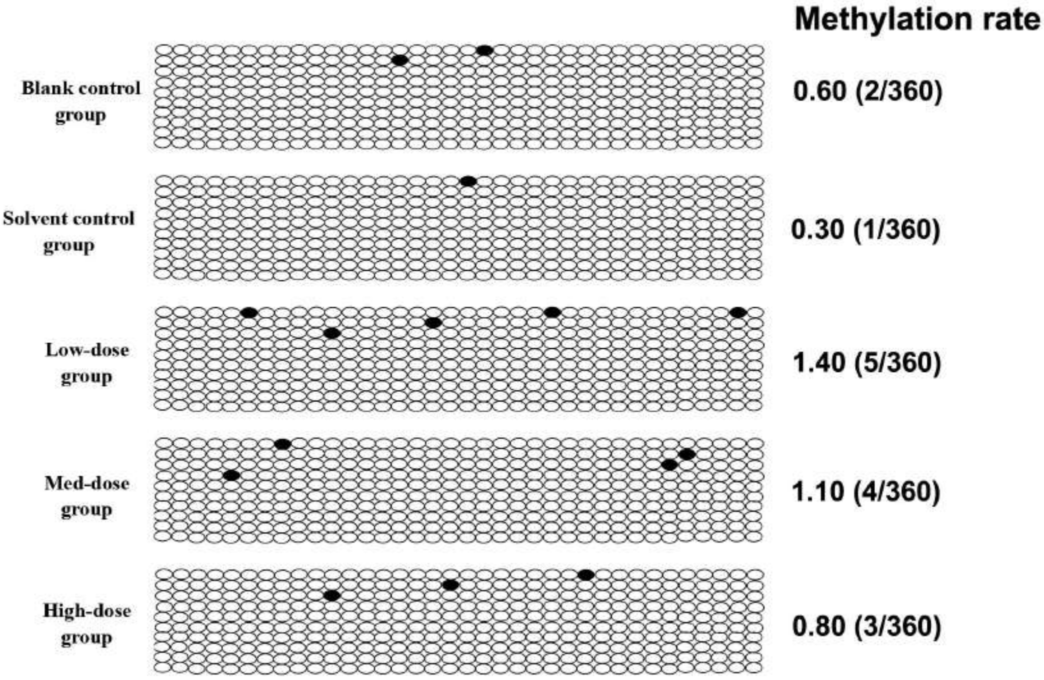


Figure 7. Results of positive control sequencing. Arrows indicate methylated CG sites.

The BSP product was sequenced using T-A cloning. After HQ exposure in K562 cells, the methylation rate among the groups did not change significantly (P>0.05) and similar results were observed in SD rats exposed to benzene (P>0.05). The results of the sequencing were plotted as shown in Figure 8a and Figure 8b; each circle represents a CG site, a solid circle represents a CG site where methylation occurred, and a hollow circle represents a CG site that did not undergo methylation.



(a)



(**b**)

Figure 8. Methylation rate of XPD gene. (**a**) Methylation rate of XPD gene after HQ exposure to K562 cells; (**b**) Methylation rate of XPD gene after benzene exposure to SD rats. ● and ○ represent methylation sites and non-methylation sites, respectively.

4. Discussion

The current study shows that benzene exposure may reduce the number of peripheral red blood cells and white blood cells, and may also lead to the damage of rat bone marrow hematopoietic tissue. This further confirms that benzene may affect blood toxicity.

After the exposure of benzene and its metabolite, HQ to SD rats and K562 cells, the OTM of DNA damage in all exposure groups was higher than in the blank control group. OTM increased with increasing benzene and its metabolite exposure concentration, indicating that both their effects on cell DNA damage were dose-dependent. This is consistent with other reported studies [12, 25, 26].

To understand whether the expression of the DNA damage repair gene, XPD, changes when the cell DNA is damaged, the expression gene´s mRNA and protein were measured in all the exposure groups. The results show that low doses of benzene and HQ exposures did not change the XPD gene expression. However, when the concentrations of benzene and HQ exposure were increased, the expression of the XPD gene was upregulated, indicating that XPD gene mRNA and protein relative expression increase only when a certain dose is attained. In our previous work, the expression of XPD mRNA and protein did not change significantly after K562 cells were exposed to HQ for 48 h. In the present study, the time of exposure was extended to 72 h, and XPD gene expression increased. This indicates that the time of HQ exposure is critical for the upregulation of XPD mRNA and protein expression. Similar conclusions were obtained from studies performed by Weidong Ji et al.[22] on human bronchial epithelial cells following nickel sulfide (NiS) treatment. The study revealed that the expression of O6-methylguanine DNA methyltransferase (MGMT) did not change following NiS treatment for 24h and 48 h but only after 72 h exposure. These results suggest that both exposure time and dose affect XPD gene expression. The possible reason may be that benzene and HQ can break DNA strands and other DNA damage, and the XPD gene is an important repair DNA damage gene. Therefore, when the cell gene is damaged, XPD gene expression increases responsively to complete the repair work for the damaged gene.

Gene expression can be affected by a variety of factors such as DNA methylation-modified epigenetic abnormalities which can change the expression of the coding gene. At present, the mechanism of DNA methylation affecting gene expression is mainly in the following two aspects: Firstly, through directly changing the genetic configuration, abnormal gene methylation interferes with specific transcription factors and recognition and binding of gene promoter region to influence gene transcription. Secondly, after methylation of gene 5' end regulatory sequence, it binds to the nuclear methyl CpG-binding protein (MeCP) to prevent transcription factors and genes from forming transcription complexes, thus indirectly affecting gene transcription [27, 28]. At the genomic methylation level, tumor cells manifest hypomethylation at the whole genome level and hypermethylation of localized regions compared with normal cells[29]. Many studies have reported that benzene and its metabolite hydroquinone can cause a decrease in the methylation level of whole genome DNA in mammalian or human cells and the whole genome methylation level is closely related to the instability of the genome[30-33]. However, the current reports about the effect of benzene and HQ on the methylation level of specific genes are very limited. No reports have been found on whether benzene and HQ can affect the methylation status of the DNA damage repair gene, XPD.

To further analyze whether the abnormal expression of the XPD gene is caused by methylation abnormalities, the present study used Methyl Primer Express v1.0 and MethPrimer online software to analyze the XPD gene promoter region. Methylated BSP primers were designed according to the methylation primer design principle[34] and the methylation level of the XPD gene was detected using the BSP method. The results showed that benzene and HQ-exposed rats and K562 cells did not cause abnormal methylation of the XPD promoter region of the nucleotide excision repair gene. The possible reasons are analyzed as follows: difference in cell species may be a factor. Suzuki R et al.[35] conducted methylation analysis of the cell inhibition factor, Dickkopf-related protein 1 (DKK1), on 5 different leukemia cells and the results showed that the methylation status of the DKK1 gene was different in different cell lines. Li Xiaoyu et al. [36] measured the methylation status of the IEX-1 gene promoter region, CpC island from 9 types of malignant hematologic disease cell lines. The results suggested that depending on the malignant hematologic disease cell line, the methylation status of the IEX-1 gene promoter region CpC island was different. In the present study, only one leukemia cell line K562 was selected for the experiment. Further studies are needed to expand the investigation scope and to select a variety of different types of leukemia cell lines to explain more accurately and objectively whether benzene and HQ exposure can cause changes in the methylation status of the XPD gene. Secondly, under the exposure of benzene and its metabolite, the methylation status of the same gene may vary [32]. For example, the research from Bollati et al. [33] showed that low concentration of benzene exposure led to p15 gene hypermethylation while Seow et al. [37] presented opposite results revealing that petroleum workers with a history of benzene exposure had hypomethylated p15 gene. All these studies suggest that the selection of a correct time point may play a crucial role in the detection of methylation status in the gene promoter region. Further studies will consider extending the exposure time to determine whether the methylation status of the XPD gene changes. Thirdly, the current study predicts that there is a CpG island in the XPD gene which was the only one studied in this investigation. However, there may be other CpG islands in the XPD gene promoter region that were not detected in this study. Finally, under the exposure of benzene and its metabolite, the expression of the gene is not necessarily caused by the methylation abnormality of the gene. Conti A et al. [31] indicated that the expressions of LINE-1, Alu and HERVs were all changed by benzene exposure, but only the methylation status of the LINE-1 gene was changed, and the methylation status of the other two genes did not change, which is consistent with the current study. These suggest that there may be other modes of regulation that affect gene expression, such as transcription factor regulation or histone methylation and acetylation changes. Future studies will select other factors that affect gene expression to analyze the reasons for XPD gene expression upregulation after benzene and HQ exposure.

5. Conclusions

In recent years, the widespread use of benzene and HQ has raised concerns about its acute and chronic toxic effects. Current studies have confirmed that benzene has a certain degree of blood toxicity and cell DNA damage. Investigation on the expression and regulation of DNA damage repair gene, XPD, is of great significance to clarify the mechanism of blood toxicity caused by benzene and HQ.

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