

# Epigenetic modification involved in benzene-induced apoptosis through regulating apoptosis-related genes expression

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## Abstract

Benzene is an established haematotoxic and genotoxic carcinogen. DNA methyltransferase inhibitor, 5-aza (5-aza-2'-deoxycytidine) and histone deacetylase inhibitor, TSA (trichostatin A) are two kinds of key epigenetic modification reagents. Although apoptosis has been considered as the key cytotoxicity mechanism, the effects of these epigenetic reagents on benzene-induced apoptosis have not been reported. In this study, BMCs (bone marrow cells) from rats were incubated with benzene and then with either 5-aza, TSA alone or the combination of the two drugs. Apoptosis and mRNA expression were detected by annexin V/PI (propidium iodide) staining assay and real-time PCR, respectively. Results showed that benzene caused cell apoptosis accompanied with bcl-2 mRNA decrease, caspase-3 and bax mRNA increase. Moreover, benzene-induced apoptosis and the decrease of bcl-2 mRNA were both reversed by both 5-aza and TSA, but the role of TSA was significantly larger than 5-aza. More interestingly, these increases in benzene-induced caspase-3 and bax mRNA expression were obviously suppressed by 5-aza but not by TSA. In conclusion, 5-aza inhibited benzene-induced apoptosis through down-regulating of caspase-3 and bax and up-regulating bcl-2 mRNA expression, whereas the effect of TSA on apoptosis dominantly affected bcl-2 mRNA expression, and 5-aza together with TSA had no synergic effect on benzene-induced apoptosis.

Keywords: 5-aza-2'-deoxycytidine; apoptosis; benzene; real-time PCR; trichostatin A

## 1. Introduction

Benzene, a ubiquitous environmental pollutant and important industrial solvent, is found mainly in unleaded gasoline, cigarette smoke and industrial emissions. The global use of benzene is now estimated to be more than 15000000 tonnes. Benzene is also a well-known human carcinogen. There are many experimental and epidemiological evidences indicating that long-term exposure to benzene is associated with haematotoxicity and haematopoietic dysfunction and is involved in the development of aplastic anaemia and leukaemia (Smith, 1996; Linet et al., 1996; Snyder, 2000; Phibbs, 2001).

Apoptosis is the most important cell death system for preventing cancer and decreasing the malignant risk. Deregulation of apoptosis contributes to tumour progression by permitting the survival of mutated cells, but its relationship with carcinogenesis has not yet been proved. Although apoptosis has been considered as one of the key cytotoxicity mechanisms induced by benzene, the underlying molecular pathways still remain unclear.

DNA methylation and chromatin structure are two modes of epigenetic control. Silencing of genes has been shown to occur by aberrant DNA methylation in the promoter region and by changes in chromatin structure that involves histone deacetylation (Herman and Baylin, 2003). It is well known that DNMT (DNA methyltransferase) inhibitor and HDAC (histone deacetylase) inhibitor are

two kinds of pivotal epigenetic modification reagents. An inhibitor of DNA methylation, 5-aza (5-aza-2'-deoxycytidine) can reverse DNA methylation patterns and has shown potent antitumour activity, suggesting its usefulness as novel cancer therapeutic agent. Similarly, TSA (trichostatin A), a potent inhibitor of histone deacetylase, can induce cell cycle arrest, apoptosis and differentiation by blocking deacetylation function. Inhibition of HDAC will activate these silenced genes, contributing to growth arrest, differentiation and apoptosis of the transformed cells (Marks et al., 2001). DNA methylation and histone deacetylation appear to act as synergistic layers for the transcriptional silencing of genes in cancer (Cameron et al., 1999; Zhu et al., 2001; Kondo et al., 2003). Epigenetic modifiers, when used alone or in combination, may currently be beneficial for various cancer patients. Recently, numerous studies have emerged, which support the combination of HDACi (histone deacetylase inhibitors) and DNA methyltransferase inhibitors (Gao et al., 2008).

Although apoptosis has been considered as the key mechanism of cell death, only a few reports have been involved in the benzene-induced deregulation of apoptosis. Effect of epigenetic modification on benzene-induced apoptosis has not been reported until today. Bone marrow is the target tissue of benzene action. Therefore, we, for the first time, examined the mechanism of apoptosis caused by benzene in BMCs (bone marrow cells) from rats.

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**Abbreviations:** 5-aza, 5-aza-2'-deoxycytidine; BMCs, bone marrow cells; CT, cycle threshold; DNMT, DNA methyltransferase; HDAC, histone deacetylase; PI, propidium iodide; TSA, trichostatin A.

## 2. Materials and methods

### 2.1. Bone marrow preparation

Under anaesthesia, femurs and tibias from male SD (Sprague–Dawley) rats were rapidly removed and cleaned of tissue. BMCs were flushed out with complete RPMI 1640 media containing 10% fetal bovine serum using a syringe and a 23- or 26-gauge needle. At least 2 million BMCs were transferred to 1.5-ml centrifuge tubes, pelleted by centrifugation and re-suspended in RPMI 1640 media containing 10% fetal bovine serum at 37°C humidified atmosphere with 5% CO<sub>2</sub> to prepare the following experiments. The details of this study were submitted to the IAEC (Institute Animal Ethics Committee) in advance, and approval of the ethics committee was obtained.

### 2.2. Cell culture and benzene treatment

BMCs isolated from the adult rat femurs and tibias were exposed to 10 mM benzene for 24 h; then, BMCs were incubated in medium containing 10 μM 5-aza (Sigma) alone or in combination with 200 nM TSA (Sigma).

### 2.3. 5-Aza and TSA treatment

Dissolved in PBS pH 6.8 was 5-aza and stored at –20°C. TSA was dissolved in absolute ethanol at a concentration of 4 mM, protected from light and stored at –20°C. BMCs were incubated with the medium containing 10 mM benzene for 24 h and then were treated with 10 μM 5-aza for 72 h or 200 nM TSA for 24 h. For combined treatment, cells were cultured with 10 μM 5-aza for 48 h and then with 200 nM TSA for another 24 h. Reagents and medium were exchanged every 24 h (Liu et al., 2005; Deng et al., 2009). These cells were then used for apoptosis analysis and total RNA isolation.

### 2.4. Apoptosis assay by annexin V/PI (propidium iodide) staining assay

Apoptosis was measured by the annexin V/PI apoptosis detection kit (BD Pharmingen) according to the manufacturer’s instructions. Briefly, the cells were harvested and washed twice with cold PBS and then resuspended in 1 × binding buffer at 1 × 10<sup>5</sup> cells/100 μl. After 5 μl of annexin V-FITC were added, the cells were incubated for 12 min at room temperature in the dark. Subsequently, the cells were pelleted and resuspended in 0.5 ml of binding buffer prior to addition of 10 μl of PI. The cells were then subjected to flow cytometry analysis using a FACScan flow cytometer (Becton Dickinson). The amount of early apoptosis and late apoptosis/

necrosis were determined, respectively, as the percentage of annexin V+/PI– or annexin V+/PI+ cells. The analysis in the flow cytometer was carried out within 1 h.

### 2.5. Analysis of mRNA expression levels by real-time PCR

Total RNA was isolated from the pooled BMCs according to the method of Gao et al. (2009). RNA integrity was confirmed by denaturing agarose gel electrophoresis, and the concentration was quantified by measuring the OD (optical density) at 260 nm. cDNA was generated from 0.2 μg of total RNA using the TaqMan® reverse transcription reagents with random hexamer primers according to the manufacturer’s protocol (Applied Biosystems Inc.). Real-time PCR was conducted in an ABI 7900 Cycle detection system using SYBR Green I as detection dye, and target cDNA was quantified using relative quantification method. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as an endogenous control to normalize expression levels. Primers for bax, bcl-2, caspase-3 and GAPDH (Table 1) were designed using Primer express soft. All reactions were assembled in 20 μl in optical 96-well reaction plates. The reaction mixture consisted of 10 μl of SYBR Green PCR master mix, 0.4 μl of ROX passive reference dye, 1 μl of gene-specific primer and 1 μl of cDNA template. PCR program was as follows: predenaturing at 95°C for 7 min, 40 cycles of denaturation at 94°C for 10 s and annealing and extension at 60°C for 1 min, then elongation at 60°C for 1 min, followed by the protocol for the melting curve: 80 cycles of 10 s with an increase of 0.5°C between each cycle from 55°C to 95°C. The specificity of the reaction was confirmed by observing a single peak at the expected T<sub>m</sub> on the melting curve analysis. All samples were run in triplicate. The relative amount of mRNA in the samples was calculated as percentage of GAPDH, using the following calculation: 2<sup>–ΔΔC<sub>t</sub></sup>. Mean CT (cycle threshold) values of triplicate samples were used for analysis. The comparative method (2<sup>–ΔΔC<sub>t</sub></sup>) was employed to calculate changes in gene expression between an experimental and calibrator (non-treated) sample. For relative quantification, the amount of target is normalized to an endogenous reference (housekeeping gene), and the calibrator is then given by 2<sup>–ΔΔC<sub>t</sub></sup>, where ΔΔC<sub>t</sub>=ΔCT (sample)–ΔCT (calibrator), the CT value indicates that fractional cycle number at which the amount of amplified target reaches a fixed threshold, and ΔCT represents the CT of the target gene subtracted from the CT of the housekeeping gene. The equation thus represents the normalized expression of the target gene in the unknown sample, relative to the normalized expression of the calibrator sample. For each gene of interest and on each plate, negative controls were samples in which cDNA was not added. For each sample, a dissociation curve was generated after completion of amplification and analysed in comparison with negative controls to

Table 1 Primers sequences for mRNA expression in rats

| Primers sequences | Forward primer             | Reverse primer            | Length | Tm |
|-------------------|----------------------------|---------------------------|--------|----|
| bax               | 5'-GTTACAGGGTTTCATCCAGG-3' | 5'-CGTGTCCACGTCAGCAAT-3'  | 178    | 55 |
| bcl-2             | 5'-CGGGAGAACAGGGTATGA-3'   | 5'-CAGGCTGGAAGGAGAAGAT-3' | 149    | 54 |
| Caspase-3         | 5'-CTGGACTGCGGTATTGAG-3'   | 5'-GGGTGCGGTAGAGTAAGC-3'  | 102    | 52 |
| GAPDH             | 5'-GCCATCAACGACCCCTTCAT-3' | 5'-TTCACACCCATCACAACAT-3' | 204    | 59 |

determine the specificity of PCR reaction. A validation experiment that demonstrated the approximately equal amplification efficiency for the target gene and housekeeping gene (GAPDH) was conducted.

## 2.6. Statistical analysis

All determinations were repeated in triplicate. Data were presented as means  $\pm$  S.D. Levels of significance for comparisons of means were performed using Wilcoxon signed-rank test for two samples or Kruskal–Wallis rank test for more samples (SPSS 12 for Windows).

## 3. Results

### 3.1. Benzene-induced apoptosis and apoptosis-related mRNA expression

BMCs were treated by 10 mM benzene for 24 h, then apoptosis and apoptosis-related genes such as caspase-3, box and bcl-2 mRNA expression were detected by annexin V/PI staining assay (Figures 1A–1B) and real-time PCR, respectively. Typical examples of the apoptosis detected by using flow cytometer are shown (Figure 1A). A significant increase in the early apoptosis percentage in benzene-treated cells compared with the control ( $P < 0.01$ ) are shown (Figure 1B). The significant increase in caspase-3 (Figure 2A) and bax (Figure 2B) and decrease in bcl-2 (Figure 2C) mRNA expression were seen in 10 mM benzene-treated cells compared with the control ( $P < 0.01$ ).

### 3.2. Effects of 5-aza on benzene-induced apoptosis

To investigate the effect of 5-aza, a pivotal epigenetic modification reagent, on benzene-induced apoptosis, the above benzene-treated BMCs were incubated with 10  $\mu$ M 5-aza for 72 h. Results indicated that the benzene-induced apoptosis was significantly inhibited by 5-aza ( $P < 0.01$ ) (Figure 1B); these increases in caspase-3 (Figure 2A) and bax (Figure 2B) mRNA expression were also obviously suppressed by 5-aza ( $P < 0.01$ ). The decrease in bcl-2 (Figure 2C) mRNA expression was reversed by 5-aza ( $P < 0.01$ ). It suggested that 5-aza inhibited benzene-induced apoptosis through down-regulating caspase-3 and bax mRNA expression and up-regulating bcl-2 mRNA expression.

### 3.3. Effects of TSA on benzene-induced apoptosis

TSA, histone deacetylase inhibitor, is another kind of key epigenetic inhibitor. The above benzene-treated BMCs were incubated by 200 nM TSA for 24 h. Results indicated that TSA attenuated the benzene-induced apoptosis ( $P < 0.05$ ) (Figure 1B). Benzene-induced decrease in bcl-2 mRNA expression was also significantly reversed by TSA ( $P < 0.01$ ) (Figure 2C), and the role of TSA was larger than that of 5-aza (Figure 2C). However, more interestingly, TSA, completely different from 5-aza, had no effect on the benzene-induced increase in caspase-3 (Figure 2A) and bax (Figure 2B) mRNA expression. It suggested that the role of TSA on benzene-induced apoptosis mostly affected bcl-2 mRNA expression.

### 3.4. Effect of 5-aza in combination with TSA on benzene-induced apoptosis

Two kinds of epigenetic inhibitors, 5-aza and TSA, were incubated with benzene-treated BMCs; the effect of 5-aza combined with TSA on benzene-induced apoptosis was not distinct (Figure 1B), but 5-aza combined with TSA was similar to 5-aza alone in suppressing caspase-3 (Figure 2A) and bax (Figure 2B) mRNA expression and in reversing bcl-2 (Figure 2C) mRNA expression ( $P < 0.01$ ). The 5-aza combined with TSA was different from TSA alone in suppressing caspase-3 (Figure 2A) and bax (Figure 2B) mRNA expression, but consistent with TSA alone in reversing bcl-2 (Figure 2C) mRNA expression ( $P < 0.01$ ).

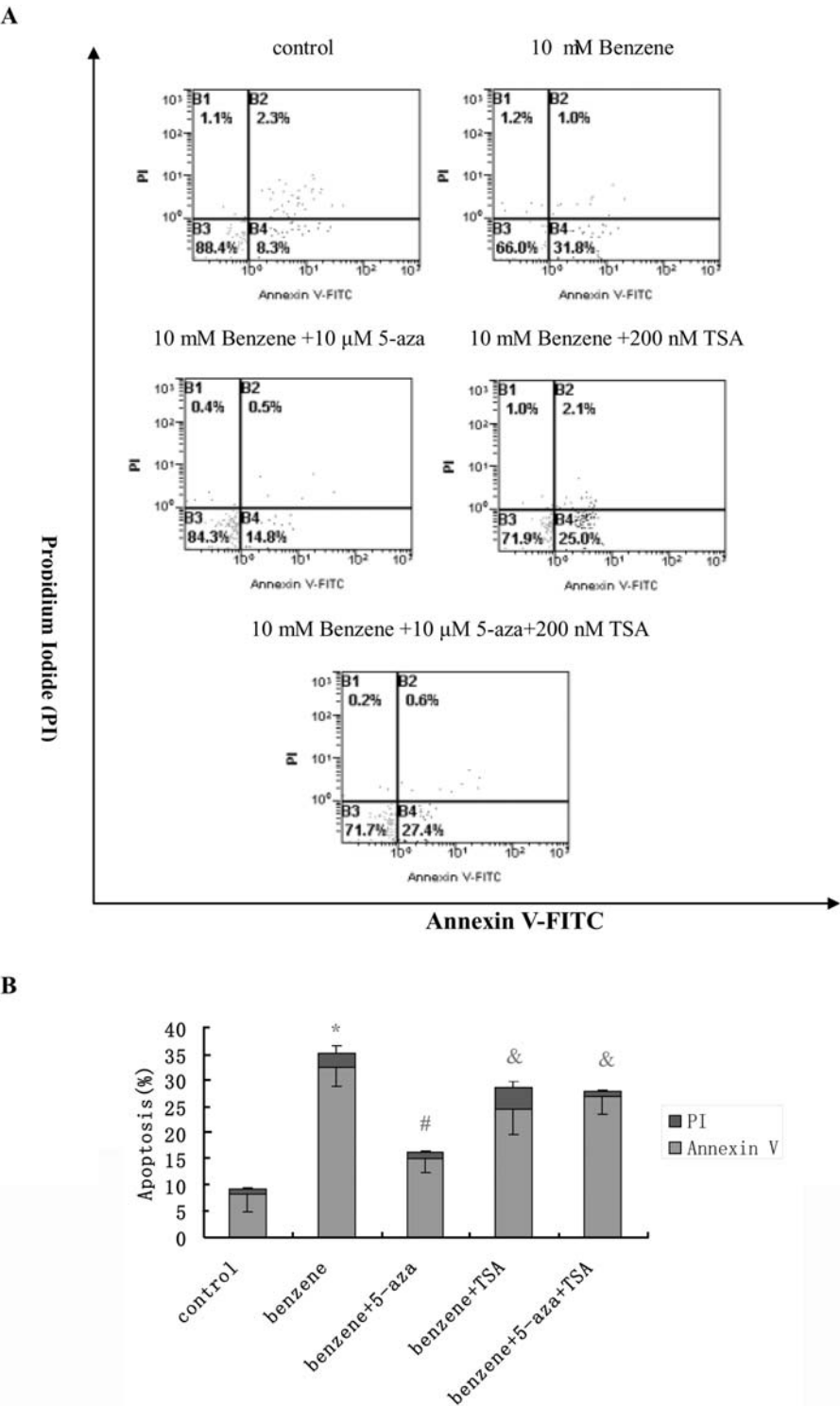
## 4. Discussion

Benzene, one of the most widely used industrial chemicals, is a widely recognized human carcinogen whose carcinogenesis effects are attributed to the production of reactive oxygen species and DNA damage. However, only a few reports were involved in the benzene-induced deregulation of apoptosis.

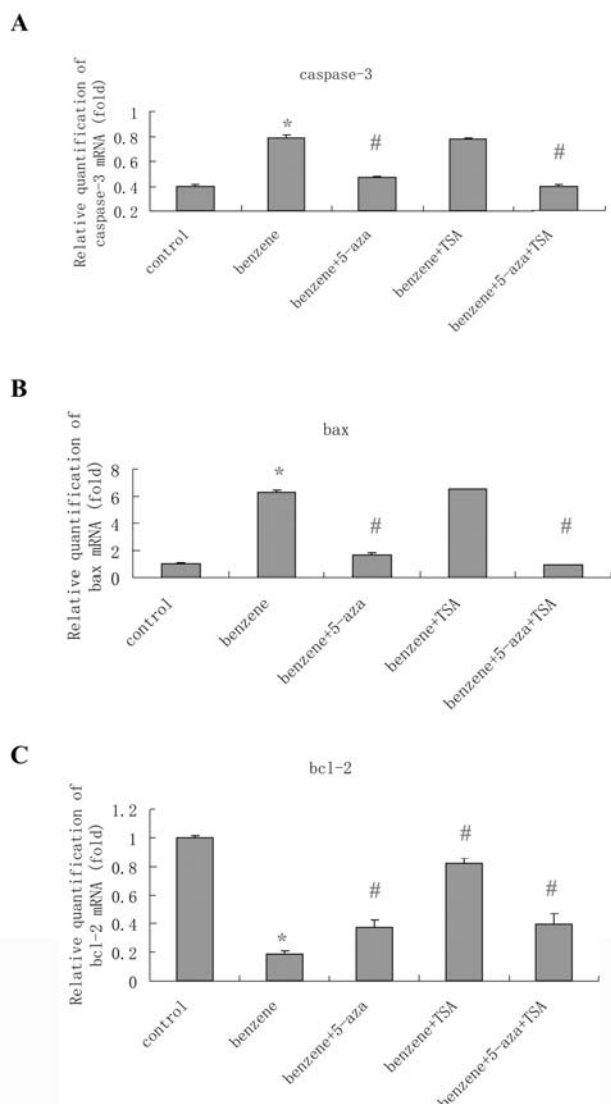
Apoptosis is an important form of cell death. The published results of benzene-induced apoptosis were still controversy for using different cell lines such as HeLa, lymphocytes and the epithelial lung cells (Martínez-Velázquez et al., 2006; Weaver et al., 2007; Galván et al., 2008). It is well known that bone marrow is the target tissue of benzene action. Therefore, we, for the first time, examined the mechanism of apoptosis effect caused by benzene in BMCs from rats. On the other hand, the effect on epigenetic modification on benzene-induced apoptosis has not been reported until today. BMCs were treated by benzene and followed by the epigenetic inhibitors including 5-aza and TSA. Apoptosis was detected by annexin V/PI staining assay using a flow cytometer. A significant increase of the early apoptosis in benzene-treated cells was shown. This result was consistent with the reports of Inayat-Hussain (2001) and Abernethy (2004).

DNA methylation and chromatin structure are two modes of epigenetic control. Although it is well established that chromatin silencing can lead to DNA methylation, the relation between chromatin activation and DNA demethylation is unclear. It is generally believed that the DNMT inhibitor 5-aza is widely used to study the re-expression of genes silenced by promoter methylation, and the methylated DNA is often associated with the deacetylated histone, which supports the essential roles of both DNMT and HDAC in silencing expression of the methylated genes. Taken together, evidence for a reversible cross-talk between histone acetylation and DNA demethylation has existed (Ou et al., 2007).

To select the best action dose of benzene, 5-aza and TSA to apply in this study, a WST-1 assay was performed to detect cell viability. Results showed that 10 mM benzene, 10  $\mu$ M 5-aza and 200 nM TSA had no cytotoxic effects when used in this study (data not shown). The appropriate time of benzene, 5-aza and TSA in our experiments was selected according to our previous assays (data not shown) and the published paper (Liu et al., 2005; Deng et al., 2009).



**Figure 1** Effects of 5-aza and TSA alone or in combination on benzene-induced apoptosis  
Apoptosis was detected by annexin V/PI staining assay using flow cytometer (A, B). (A) Typical example of three independent experiments. The x-axis represented the annexin V labelling, while the y-axis represented the PI staining. (B) Benzene+5-aza, benzene+TSA and benzene+5-aza+TSA denoted that benzene-treated cells were incubated with 10  $\mu$ M 5-aza for 72 h, 200 nM TSA for 24 h, 10  $\mu$ M 5-aza for 48 h and then with 200 nM TSA for another 24 h, respectively. \* $P$ <0.01, compared with the control group, # $P$ <0.01, compared with the benzene treatment group, & $P$ <0.05, compared with the benzene treatment group,  $n$ =3.



**Figure 2** Effects of 5-aza and TSA alone or in combination on benzene-induced caspase-3 (A), bax (B) and bcl-2 (C) mRNA expression by real-time PCR

Benzene+5-aza, benzene+TSA and benzene+5-aza+TSA denoted that benzene-treated cells were incubated with 10  $\mu$ M 5-aza for 72 h, 200 nM TSA for 24 h, 10  $\mu$ M 5-aza for 48 h and then with 200 nM TSA for another 24 h, respectively. \* $P$ <0.01, compared with the control group, # $P$ <0.01, compared with benzene treatment group,  $n$ =3.

In this report, the epigenetic inhibitors 10  $\mu$ M 5-aza and 200 nM TSA, alone or in combination, were further used to incubate benzene-treated cells. Results showed that the epigenetic modification was involved in benzene-induced early apoptosis. To my knowledge, the result was first reported.

Caspase-3, bax and bcl-2 have been proven to play a pivotal role in mitochondria-dependent apoptosis (Cory and Adams, 2002; Lim et al., 2002; Rudner et al., 2002). To confirm whether benzene induced cell apoptosis through apoptotic-related genes and to further clarify the above detailed action mechanism, we investigated whether apoptosis-related genes, such as caspase-3, bax and bcl-2 were also regulated by the epigenetic inhibitors. BMCs from rats were first treated by benzene and followed by the

epigenetic inhibitors such as 5-aza and TSA. These apoptosis-related genes were monitored by real-time PCR. A significant increase in caspase-3 and bax mRNA expression was seen in benzene-treated cells compared with the control, and the epigenetic inhibitors, 10  $\mu$ M 5-aza alone or in combined with 200 nM TSA, either suppressed the above phenomenon, 200 nM TSA alone cannot inhibit caspase-3 and bax mRNA expression increases. A dramatic decrease in bcl-2 mRNA expression in benzene-treated cells was shown, and the epigenetic inhibitors, 10  $\mu$ M 5-aza and 200 nM TSA, alone or in combination, both reversed the decrease, but the role of TSA was larger than 5-aza. Furthermore, 5-aza, together with TSA, had no synergic effect on benzene-induced apoptosis. These findings suggested that two kinds of epigenetic modification reagents were involved in benzene-induced apoptosis through affecting different apoptosis-related genes, respectively.

### Author contribution

All authors read and approved the final manuscript. Ai Gao performed the study design, analysis, interpretation of data, manuscript drafting and critical discussion. Xin Zuo performed the experiment, or acquisition of data, statistic analysis. Shanshan Song and Wei Guo participated in the critical discussion. Lin Tian revised it critically and gave the final approval of the version to be published.

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