Original Article

Formaldehyde induces toxic effects and regulates the expression of damage response genes in BM-MSCs

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In this study, we assessed the toxic effects of formaldehyde (FA) on mouse bone marrow mesenchymal stem cells (BM-MSCs). Cytotoxicity was measured by using MTT assay. DNA strand breakage was detected by standard alkaline comet assay and comet assay modified with proteinase K (PK). DNA–protein crosslinks (DPCs) were detected by KCl-SDS precipitation assay. We found that FA at a concentration from 75 to 200 μM inhibited cell survival and induced DPCs over 125 μM. The PK-modified comet assay showed that FA-induced DNA strand breakage was increased in a dose-dependent manner from 75 to 200 μM. On the other hand, standard alkaline comet assay showed that DNA strand breakage was decreased with FA concentration over 125 μM. We confirmed by using Pearson correlation that there was a negative linear correlation between DPCs and survival rate (r = −0.987, P < 0.01) and positive linear relationships between DPCs and (i) sister chromatid exchange (r = 0.995, P < 0.01; r = 0.968, P < 0.01). DNA damage RT2 profiler polymerase chain reaction array was used to investigate the changes in the expression of damage response genes. Xpa and Xpc of the nucleotide excision repair pathway and Brca2, Rad51, and Xrc2 of the homologous recombination pathway were all up-regulated in both 75 and 125 μM FA. However, the same genes were down-regulated with 175 μM FA. The expressions of Chek1 and Hus1, which are involved in cell cycle regulation, were altered in the same manner with 75, 125, and 175 μM FA. These results indicated that Xpa, Xpc, Brca2, Rad51, Xrc2, Chek1, and Hus1 were essential for the BM-MSCs to counteract the effects of FA.

Keywords comet assay; DNA–protein crosslinks; sister chromatid exchange; micronuclei; PCR array

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Introduction

Formaldehyde (FA), an aliphatic monohaldehyde, is widely present in automobile emissions, tobacco smoke, and various household products [1,2]. In 2012, the International Agency for Research on Cancer (IARC) re-evaluated FA as a known human carcinogen (group 1) that causes leukemia and cancer of the nasopharynx; however, the mechanisms remain unclear [3]. FA is also naturally present in all tissues and bodily fluids. FA is an intermediate in the ‘one-carbon pool’ used for the biosynthesis of purines, thymidine, certain amino acids, and the demethylation of various important biological compounds that are central to cell function and survival. Heck and Casanova [4] demonstrated that the background levels of endogenous FA in human plasma range from 13 to 97 μM. Endogenous FA is usually rapidly metabolized by reduction, oxidation, and reduced glutathione-dependent pathways. However, excess FA, including that encountered occupationally and/or environmentally, could exceed normal capacity for metabolism and lead to cytotoxicity and genotoxicity.

In aqueous solution, FA maintains dynamic equilibrium with its diol form, i.e. methanediol [formaldehyde hydrate, CH2(OH)2, or methylene glycol]. More importantly, methanediol, with a molecular weight of 48 g/mol, can readily penetrate into tissue. Thus, FA can reach the bone marrow (BM) through the blood as methanediol, where it converts back to reactive FA [5]. Several studies have reported that the level of cytogenetic damage increases in the BM of exposed mice and rats [6,7]. Zhang et al. [8] also reported that FA-exposed workers had lower blood counts and...
increased chromosomal damage degree in myeloid blood progenitor cells. These findings suggest that FA can reach and exert a toxic effect on BM.

As one component of BM, bone marrow mesenchymal stem cells (BM-MSCs) are notable for their multi-differentiation potential. Also, BM-MSCs are crucial for maintaining tissue homeostasis [9,10]. A recent study suggested that BM-MSCs and hematopoietic stem cells form a unique BM niche, and BM-MSCs help hematopoietic stem cells in maintaining function and their primary properties [11,12]. Thus, we hypothesized that if FA induces genotoxicity and mutagenicity in BM-MSCs, BM-MSCs will lose their normal control over hematopoietic stem cells, leading to myeloid leukemia. However, to date, the ability of FA exposure to disrupt BM-MSCs has not been reported.

FA exhibits genotoxicity and mutagenicity in various kinds of mammalian cells, including cultured human myeloid progenitor cells and V79 Chinese hamster cells [2,13,14]. It is generally accepted that the primary DNA damage induced by FA results in DNA–protein crosslinks (DPCs). The level of FA-induced DPCs is considered to be a good molecular dosimeter to assess FA-related cancer risk [15]. However, there has always been a debate on whether DNA strand breakage was induced by FA.

There is a complex cellular response to FA exposure. Several studies have mentioned specific cellular activities used to counteract FA, such as nucleotide excision repair (NER) [16–18], proteasomal degradation [19], Fanconi/BRCA pathway [20], and homologous recombination (HR) [20,21]. However, there are few systematic and comprehensive studies characterizing changes in damage response genes in cells exposed to FA.

The present study was performed to characterize the cytotoxic and genotoxic effect of FA on mouse BM-MSCs. Subsequently, we used the RT² profiler polymerase chain reaction (PCR) array to obtain the expression profile of damage response genes and comprehensively assess the DNA repair, cell cycle, and apoptosis pathways in BM-MSCs that are used to counteract the effects of FA.

Materials and Methods

Cell culture

Mouse BM-MSCs, purchased from the American Type Culture Collection (CRL-12424; Manassas, USA), were cultured in Dulbecco’s modified Eagle’s medium (DMEM)/F-12 (1:1) supplemented with 10% fetal calf serum (all from HyClone Perbio, Erembodegem, Belgium), penicillin (50 U/ml), and streptomycin (50 μg/ml) at 37°C in a humidified incubator with 5% CO₂. For the experiments, the BM-MSCs were detached with 0.25% Trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco, Paisley, UK) and randomly divided into the control group and various experimental groups 24 h prior to FA exposure. FA (CAS No. 50-00-0; 37%) was purchased from Sigma (St Louis, USA). Experimental groups included 25, 50, 75, 100, 125, 150, and 200 μM FA treatment groups for MTT assay; 75, 100, 125, 150, 175, and 200 μM FA treatment groups for Comet, KCl-SDS precipitation, sister chromatid exchange (SCE), and micronucleus tests (MNTs); and 75, 125, and 175 μM FA treatment groups for RT² profiler PCR array. The cells in the control group were treated with FA-free DMEM/F-12 and those in the experimental groups were treated with FA at appropriate concentrations of DMEM/F-12. FA was changed every 6 h.

MTT assay

BM-MSCs (4 × 10³ cells) were seeded into 96-well plates and cultured for 24 h. The medium was replaced with fresh DMEM/F-12 or the same media containing 25, 50, 75, 100, 125, 150, 175, or 200 μM FA. After incubation for 12 h, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added to each well and incubated for 4 h. The medium was then discarded and replaced with 150 μl of dimethyl sulfoxide in each well. The cultures were then incubated for 10 min. The OD₅₇₀nm was measured and the survival rate was calculated according to the formula: (experimental OD value/control OD value) × 100%. The MTT assay was repeated at least three independent times.

Comet assay

standard alkaline comet assay modified with proteinase K (PK; Sigma) was used to detect FA-induced DNA strand breakage, as described previously [15,22,23]. Briefly, after treatment with 75, 100, 125, 150, 175, or 200 μM FA for 12 h, the cells were harvested and resuspended in ice cold phosphate-buffered saline (PBS). Approximately 1 × 10³ cells in a volume of 100 μl of 1% (w/v) low melting point agarose were pipetted onto microscope slides (with frosted ends), which had been covered with a layer of 1% agarose and allowed to solidify on ice for 10 min. The slides were immersed for 2 h in ice-cold lysis solution containing 100 mM Na₂-EDTA and 2% (w/v) N-lauroyl-sarcosine solution (Sigma) that was adjusted to pH 10.0. PK treatment was conducted by using lysis solution containing 100 mM Na₂-EDTA, 2% (w/v) N-lauroyl-sarcosine, and 40% PK, which was immediately added before use, overnight at 37°C. After lysis, the slides were washed in 1× TBE buffer (pH8.3, 89 mM Tris, 89 mM boric acid, and 2.5 mM EDTA) for 10 min, then placed into a horizontal electrophoresis tank filled with buffer (pH 13, 0.3 M NaOH, and 1 mM EDTA) for alkali denaturation for 30 min. Electrophoresis was then performed for 25 min at 300 mA. The slides were transferred to and washed in neutralization solution (0.4 M Tris-HCl, pH 7.5) three times, for 5 min each wash. The samples were then fixed in H₂O₂ for 5 min and washed in double distilled water three times, for 5 min each time.
Finally, the slides were stained with 5 μg/ml propidium iodide (Sigma) for 15 min. The images were captured by using a fluorescent microscope (IX81; Olympus, Tokyo, Japan) with ×40 objective and analyzed by using CASP image analysis software (version 1.2.3; Bio-rad Laboratories, Hertfordshire, UK). The degree of DNA breakage was quantified by measuring the olive tail moment (OTM). OTM is defined as the product of tail extension and the fraction of total DNA in tail, and is calculated according to the formula: (tail mean – head mean) × (tail DNA%/100) [24]. Fifty randomly selected cells from each slide were measured by image analysis. The assay was repeated thrice.

KCl–SDS precipitation assay
DPC formation was measured based on fractionation of bound protein and free DNA by potassium-SDS precipitation [25]. Briefly, after treatment with 75, 100, 125, 150, 175, or 200 μM FA for 12 h, the cells were harvested and lysed with 0.5 ml of 2% SDS and mild oscillation at 65°C for 10 min. We then added 0.5 ml of 200 mM KCl and 20 mM Tris (pH 7.5) and kept the mixture at 65°C for 10 min. The mixture was next placed on ice for 5 min and then centrifuged at 6000 g for 5 min at 4°C. The mixture was then washed. The cycle of centrifugation and washing was repeated three times. Each time the supernatant was collected into tubes for quantification of free DNA content resulting from each treatment. The pellets were resuspended in 200 mM KCl in 20 mM Tris–HCl (pH 7.5), heated at 65°C for 10 min, incubated with 0.2 mg/ml PK at 50°C for 3 h, cooled, and centrifuged at 12,000 g for 10 min at 4°C. The supernatant was collected to determine the amount of cross-linked DNA. The amount of DNA in the supernatant was determined by Hoechst 33258 (Sigma) by measuring excitation at 350 nm and emission at 450 nm by using a Shimadzu RF-5301PC Spectrofluorometer (Shimadzu, Tokyo, Japan). The DPC level was quantified by determining the amount of cross-linked DNA as a percentage of total DNA. We calculated this value according to the formula: [cross-linked DNA/(cross-linked DNA + free DNA)] × 100%. The KCl–SDS precipitation assay was repeated three times.

SCE test
SCE test was conducted according to Merk and Speit [26]. After treatment with 75, 100, 125, 150, 175, or 200 μM FA for 12 h, the experimental medium was replaced with complete medium supplemented with 10 μg/ml 5-bromodeoxyuridine. The cells were cultivated for 24 h and colcemid (3 × 10⁻⁷ M) was added and incubated for final 2 h. Chromosome preparation was performed according to the following standard procedures. Briefly, the cells were centrifuged, resuspended in 0.4% KCl for 20 min, and fixed three times in a solution of methanol and glacial acetic acid (3 : 1). To differentiate between sister chromatids, air-dried slides were coated with Sorensen’s phosphate buffer (pH 6.8) and irradiated with an 8W UV lamp (254 nm) at a distance of 10 cm for 30 min. Subsequently, the slides were incubated in 2× saline-citrate buffer for 20 min at 60°C and then stained with 7% Giemsa in Sorensen’s phosphate buffer. SCE was scored in 30 cells per sample from the coded slides. The SCE test was repeated thrice.

Micronucleus test
The MNT was conducted as described previously with minor modifications [27]. Briefly, after treatment with 75, 100, 125, 150, 175, or 200 μM FA for 12 h, the BM-MSCs were detached by trypsin, treated with a hypotonic solution (0.56% KCl), and fixed with methanol/glacial acetic acid (5 : 1) mixed with an equal amount of 0.9% NaCl. The cells were then treated three more times with methanol/glacial acetic acid. The air-dried slides were stained with 10% Giemsa in PBS. The amount of micronuclei (MNs) present was determined by analyzing 1000 cells from the coded slides. The MNT was repeated thrice.

Total RNA extraction and reverse transcription
The cells were incubated with 75, 125, or 175 μM FA for 12 h. The solutions were replaced with 1.0 ml TRIzol (Invitrogen Life Technologies, Shanghai, China) and chloroform. The samples were vigorously vortexed for 15 s then incubated at room temperature for 5 min. They were then subjected to centrifugation at 12,000 g for 30 min at 4°C. We transferred the upper aqueous phase to a fresh tube, added equal amounts of cold isopropanol, and incubated the samples at room temperature for 10 min. They were then subjected to centrifugation at 12,000 g for 10 min at 4°C. After the RNA precipitated out, it was washed in cold 70% ethanol, air-dried, and then resuspended in 25 μl of RNase-free H₂O. RNA quality was determined by running a 1% agarose gel and inspecting for distinct 18S, 28S, and total RNA bands, which showed the lack of degradation. A BIOMATE 3S UV-VISIBLE Spectrophotometer (Thermo Scientific, Madison, USA) was used to determine RNA quality. All of the samples were stored at −80°C.

Real-time PCR-based array analysis
First-strand cDNA synthesis was performed with 1 μg of total RNA and RT² First Strand Kit (QIAGEN Sciences, Maryland, USA) according to the manufacturer’s instructions. Briefly, the diluted first-strand cDNA synthesis react-ant was mixed with RT² SYBR® Green qPCR Mastermix (QIAGEN Sciences) and pipetted into 96-well PCR array plates (RT² Profiler PCR Array, mouse DNA repair; QIAGEN Sciences) to evaluate the expression of 84 DNA damage response genes. Real-time PCR detection was
performed in a cycler (Bio-Rad CFX96™; Applied Biosystems,
Singapore) by heating the plate at 95°C for 10 min, followed by
40 cycles of 95°C for 15 s and 60°C for 1 min. The CT data
were uploaded using a PCR array data analysis spreadsheet
template on the PCR Array Data Analysis Website at
www.SABiosciences.com/pcrarraydataanalysis.php. At this
website, the web-based software automatically quantified
gene expression using the ΔΔCT method. Quality controls
confirmed the lack of DNA contamination and were success-
fully tested for RNA quality and PCR performance.

Housekeeping genes Gusb, Hprt, HSP90ab1, Gapdh, and
Actb were used to normalize data. After normalization, the
relative expression level of each gene was averaged for the
three samples in each concentration group. The assay was
repeated twice.

We identified genes that were differentially expressed in
BM-MSCs after exposure to FA in comparison with the
control using a two-sample t-test. Gene expression was con-
sidered statistically significant if their P-value was less than
0.05 and the change was 2-fold between the two groups.

Statistical analysis
Data were expressed as mean ± SD. SPSS 17.0 for
Windows (SPSS Inc., Chicago, USA) was used to analyze
data. One-way analysis of variance followed by Tukey’s or
Tamhane’s T2 post hoc test was used to evaluate the statisti-
cal significance of differences among the groups. In addi-
tion, we used the Student’s t-test to compare (i) gene
expression in the FA-treated group with that of the control
and (ii) DNA strand breakage found using the standard
alkaline comet assay with that found via the comet assay
modified with PK. The relationships between DPCs and
(i) cytotoxicity, (ii) SCE and (iii) MN frequency were deter-
mined by Pearson correlation. P < 0.05 was considered stat-
istically significant.

Results

Cytotoxicity of FA to BM-MSCs
MTT assay was used to determine the cytotoxicity of FA to
BM-MSCs by calculating the survival rates after the cells
were treated with different concentrations of FA for 12 h.
The results showed that cytotoxicity was observed in 75–
200 μM FA (P < 0.01) and increased gradually as FA con-
centration increased [Fig. 1(A)]. The survival rates of cells
treated with 75, 100, 125, 150, 175, and 200 μM FA were
94.533%, 91.752%, 87.547%, 81.652%, 66.068%, and
53.023%, respectively. When the cells were exposed to 175
or 200 μM FA, the increase in cytotoxicity was remarkable.
Therefore, an FA concentration ranging from 75 to 200 μM
was used in subsequent genotoxicity experiments.

Alterations of the surface of BM-MSCs were clearly
observed under a phase-contrast microscope after treating
cells with FA at concentrations of 125 μM or above. The
control BM-MSCs displayed spindle-shaped, flat, fibroblast-
like morphologies and were arranged in a spiral shape. On
the other hand, the BM-MSCs exposed to FA were
round, smaller, and arranged less in a spiral shape. Several
BM-MSCs also exhibited plasma membrane blebbing
[Fig. 1(B)].

Induction of DNA strand breakage in BM-MSCs by FA
In order to identify DNA strand breakage in cells, we carried
out the standard alkaline comet assay and the comet assay
modified with PK on BM-MSCs exposed to FA at concen-
trations from 75 to 200 μM. DNA strand breakage was
quantified and expressed as the OTM.

The standard alkaline comet assay showed that the magni-
tude of FA-induced DNA strand breakage increased gradual-
ly at increasing concentrations below 125 μM [Fig. 2(A)].
However, when the FA concentration was over 125 μM, the
OTM in BM-MSCs decreased. The mean OTM at 175 μM
or 200 μM (24.17) was lower than that of control (26.05), and showed no significant differences (P > 0.05).

The comet assay modified with PK showed that the magnitude of DNA strand breakage increased in a dose-dependent manner after the BM-MSCs were treated with FA concentrations increasing from 75 to 200 μM. Compared with those in standard comet assay, the OTMs were all higher, but not significantly different at 75 μM (36.56 vs. 37.63) and 100 μM FA (61.31 vs. 63.77) (P > 0.05). FA-induced DNA strand breakage in the BM-MSCs was confirmed by the comet structure [Fig. 2(B)].

**Induction of DPCs in BM-MSCs by FA**
To determine the effect of FA on DPC formation in BM-MSCs, KCl-SDS precipitation assay was used. FA (from 125 to 200 μM) induced the formation of significantly more DPCs in BM-MSCs than in control (P < 0.01) (Fig. 3). The mean amount of DPCs calculated was 3.320, 7.623, 11.268, 20.457, and 24.122 in BM-MSCs treated with 0 (control), 125, 150, 175, and 200 μM FA, respectively.

**Induction of SCE in BM-MSCs by FA**
SCE was significantly induced in BM-MSCs by FA at concentrations from 125 to 200 μM (Fig. 4) (P < 0.01). SCE level in cells treated with 175 or 200 μM FA was remarkably higher than that in control (P < 0.01).

**Induction of MN frequency in BM-MSCs by FA**
FA at concentrations below 125 μM failed to induce MN frequency (Fig. 5). The frequency of cells with MNs increased from 3.777 per 1000 (control) to 41.444 per 1000 cells (at 200 μM FA). The MN frequency in cells treated with FA at concentrations from 150 to 200 μM was significantly higher than that in control (P < 0.05).

**Relationships between DPCs and cytotoxicity, SCE, and MN frequency induced by FA**
DPCs are considered the direct damage to DNA that can be caused by FA. Unrepaired DPCs can arrest DNA replication so as to induce other genotoxic effects, such as SCE and MN frequency. They can also induce cell death [26,28].
In this study, DPC formation increased in BM-MSCs due to FA at concentrations from 125 to 200 μM. Cytotoxic effect, as determined by measuring reduction in survival rate, gradually increased as FA concentration increased from 75 to 200 μM. SCE and MN frequency were induced by 125 and 150 μM FA, respectively. Moreover, these toxic effects were all significantly more severe with 175 and 200 μM FA treatments. The scatters (not shown) further indicated that the relationships between DPCs and cytotoxicity, SCE, and MN frequency can described by Pearson correlation. The calculated Pearson correlation coefficients (r) and associated P values were −0.987 and 0.000 for the survival rate; 0.995 and 0.000 for SCE; and 0.970 and 0.001 for MN frequency. These results suggest that there is a negative linear correlation between DPCs and survival rate, and positive linear relationships between DPCs and SCE as well as DPCs and MN frequency.

Changes in the expression of DNA damage response genes induced by FA

Given that FA treatment resulted in cytotoxicity and genotoxicity in BM-MSCs, we further investigated the impact of 75, 125, and 175 μM FA on the expression profile of 84 genes in BM-MSCs related to DNA damage response with RT² profiler PCR array. Figure 6 showed the scatter diagram of gene expression changes in BM-MSCs treated with 75, 125, or 175 μM FA. Table 1 summarized the significant changes in gene expression in BM-MSCs exposed to 75, 125, or 175 μM FA as determined by the PCR array analyses. The number of significantly altered genes was between 20 and 30. The individual genes were listed in Table 2. There was no correlation between the number of altered genes and the concentration of FA. In the experiments involving treatment with 75 and 125 μM FA for 12 h, it was obvious that more genes were up-regulated than down-regulated. However, treatment with FA at high concentrations (175 μM) resulted in the majority of genes being down-regulated, with only five genes up-regulated at least 2 fold. Interestingly, *Xpa* and *Xpc* of NER pathway, and *Brca2*, *Rad51*, and *Xrcc2* of the HR pathway were
up-regulated under treatment with 75 or 125 \( \mu \)M FA, whereas these genes were down-regulated under treatment with 175 \( \mu \)M. In addition, treatment with 75, 125, and 175 \( \mu \)M FA altered the expression of Chek1 and Hus1, genes involved in cell cycle regulation, in the same manner.

### Discussion

FA is highly reactive and has cytotoxic and genotoxic effects. The cytotoxicity of FA has been confirmed in numerous in vitro systems [20,29,30]. In particular, the effects of FA on growth inhibition of human blood cells and myeloid progenitor cells were observed at FA concentrations from 100 to 200 \( \mu \)M. This range is toxicologically relevant since the background levels of FA in human plasma are 13–97 \( \mu \)M. In the present study, we found that cytotoxicity in mouse BM-MSCs gradually increased as FA concentrations increased from 75 to 200 \( \mu \)M. However, the treatment of cells for 12 h was longer than that in most reported studies, which might be due to the fact that the BM-MSCs are more resistant to DNA damaging agents.

We used standard alkaline comet assay and comet assay modified with PK to detect DNA strand breakage. The

![Figure 6 RT² profiler PCR arrays revealed altered genes in BM-MSCs treated with 75, 125, or 175 \( \mu \)M FA for 12 h](https://academic.oup.com/abbs/article-abstract/45/12/1011/1120)

<table>
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<th>Treatment (( \mu )M)</th>
<th>Number of altered genes</th>
<th>Up-regulated genes*</th>
<th>Down-regulated genes*</th>
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<tr>
<td>75</td>
<td>22</td>
<td>20</td>
<td>2</td>
</tr>
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<td>125</td>
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<td>17</td>
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<tr>
<td>175</td>
<td>30</td>
<td>5</td>
<td>25</td>
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*\( P < 0.05 \) and \( \geq 2 \)-fold change.
<table>
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<tr>
<th>Treatment (µM)</th>
<th>Functional group</th>
<th>Symbol of up-regulated genesa</th>
<th>Symbol of down-regulated genesa</th>
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<tr>
<td>75</td>
<td>Nucleotide excision repair</td>
<td>Dclre1a, Trp53(P53), Xpa, and Xpc</td>
<td>Smc3</td>
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<td></td>
<td>Base excision repair</td>
<td>Apex1 and Fen1</td>
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<td>Mismatch repair</td>
<td>Mlh1</td>
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<td>Homologous recombination</td>
<td>Brca1, Brca2, Mre11a, Rad51, and Xrcc2</td>
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<tr>
<td></td>
<td>Non-homologous end joining</td>
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<td></td>
<td>Other genes related to DNA repair</td>
<td>Cry2, Faneg, Smc1a, Rad17, and Rev1</td>
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<td></td>
<td>Apoptosis</td>
<td>Chaf1a, Chek1, and Hus1</td>
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<td>Pinx1</td>
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<td>Cell cycle</td>
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*aP < 0.05 and ≥2-fold change.
standard alkaline comet assay showed that the magnitude of DNA strand breakage increased gradually at FA concentrations below 125 μM. When the formation of DPCs increased with increasing concentrations of FA over 125 μM, the magnitude of DNA strand breakage began to decrease. Therefore, we suggest that DNA migration following strand breakage in the alkaline comet assay might be inhibited by DPCs that occur at FA concentrations over 125 μM. These observations were consistent with the findings of Liu et al. [31]. PK can resolve cross-linked protein in DPCs and enhance DNA migration resulting from DNA strand breaks. Thus, we used comet assay modified with PK to study whether DNA strand breaks might not be detected due to the cross-linking effect of FA. Post-treatment with PK enhanced DNA migration in controls and FA-treated cells, and decreased the cross-linking effect of FA. Taken together, we suggest that FA induces strand breakage in a dose-dependent manner from concentrations from 75 to 200 μM. In addition, the comet assay modified with PK may be a suitable method for the detection of FA-induced DNA strand breakage.

DPCs are considered the direct result of damage to DNA that can be caused by FA [26]. In normal cells, DPCs exist at a basic level, since they are necessary for DNA replication, transcription, and cell growth. However, excessive DPCs produced by environmental pollutants and carcinogens can be highly toxic. Unrepaired DPCs can inhibit DNA replication, lead to the induction of other genotoxic effects, such as SCE [3,32], and induce cell death [28]. Incomplete repair of DPCs can lead to the formation of mutations [6] and chromosomal effects such as MN frequency [3,7]. In this study, DPCs formation were significantly increased in BM-MSCs by FA at concentrations ranging from 125 to 200 μM. The cytotoxic effect, as measured by reduction of survival rate, gradually increased as FA concentration increased from 75 to 200 μM. SCE and MN were induced by ≥125 and ≥150 μM FA, respectively. Pearson correlation analysis also confirmed that there was a negative linear correlation between DPCs and survival rate \( r = -0.987, P < 0.01 \) and positive linear relationships between DPCs and SCE \( r = 0.995, P < 0.01 \), as well as DPCs and MN frequency \( r = 0.968, P < 0.01 \). All of these observations suggest that DPCs seem to be the most relevant DNA damage induced by FA. However, these conclusions need further studies.

When FA induces DNA damage, such as DPCs, the cell starts repair mechanisms to reverse the damage to re-establish stability of the genome. The NER pathway is an important pathway for DNA damage repair, which mainly repairs the cyclobutane pyrimidine dimer, DNA adducts, and cross-linking between chains [33,34]. HR refers to the recombination of homologous DNA sequences, which mainly uses the homology of DNA sequences for recognition. The HR pathway commonly repairs DNA double-strand breakage. Increasing evidence has suggested that the fracture after excision might be linked by HR [20]. In the present study, we suggested that both ways were responsible for the repair of DNA damage caused by FA exposure. When 75 or 125 μM FA was administered, the expressions of some important genes in the NER and HR pathways, such as Apa, Apc, Brc2, Rad51, and Xrc2, were up-regulated. However, when treated with 175 μM FA, many repair genes, including these were down-regulated. This result indicates that high concentrations of FA induce DNA damage that outweighs the capacity for self repair, which, in turn, inhibits the expression of such repair genes. Subsequently, the DPCs fail to be repaired in time, resulting in the arrest of DNA replication, the mutation of chromosomes, as well as cell death [26,28], which is supported by our toxicity assay. When 175 μM FA was administered, DPC formation was significantly increased. In turn, SCE, MN frequency, and cytotoxicity induced by DPCs were significantly increased. In addition, after damage was done to the DNA, the control mechanism was activated. Essentially, the cell cycle was paused to allow sufficient time to repair the damage so as to keep the genome stable. In the present study, we found that, after treatments with 75, 125, and 175 μM, the expression levels of repair-related cell cycle control genes Chek1 and Hus1 were increased. These genes are responsible for blocking proliferation. All of these observations indicate that the NER and HR pathways, together with cycle control genes Chek1 and Hus1, are responsible for DNA repair.

In conclusion, FA is cytotoxic and genotoxic to mouse BM-MSCs in vitro. The comet assay modified with PK may be a suitable method for the detection of DNA strand breakage that increases in a dose-dependent manner from 75 to 200 μM FA. Formation of DPCs rose with FA at concentrations above 125 μM. Moreover, DPCs seem to be the most relevant DNA damage induced by FA. The expression and regulation of Xpa and Xpc of NER pathway; Brc2, Rad51, and Xrc2 of the HR pathway; and Chek1 and Hus1 involved in cell cycle regulation were essential for DNA repair in BM-MSCs after treatment with FA. These results may provide some new insights into the effect of FA on leukemogenesis. However, further research is still needed to determine if the BM-MSCs losing normal control of hematopoietic stem cells can cause myeloid leukemia.

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