

## RESEARCH ARTICLE

# Inhaled Formaldehyde Induces Bone Marrow Toxicity via Oxidative Stress in Exposed Mice

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### Abstract

Formaldehyde (FA) is an economically important chemical, and has been found to cause various types of toxic damage to the body. Formaldehyde-induced toxic damage involves reactive oxygen species (ROS) that trigger subsequent toxic effects and inflammatory responses, which may increase risk of cancer. Therefore, in the present study, we aimed to investigate the possible toxic mechanism in bone marrow caused by formaldehyde. In accordance with the principle of randomization, the mice were divided into four groups of 6 mice per group. One group was exposed to ambient air and the other three groups were exposed to different concentrations of formaldehyde (20, 40, 80 mg/m<sup>3</sup>) for 15 days in the respective inhalation chambers, 2h a day. At the end of the 15-day experimental period, all mice were killed. Bone marrow cells were obtained. Some of those were used for the determination of blood cell numbers, bone marrow karyote numbers, CFU-F, superoxide dismutase (SOD) activity and malondialdehyde (MDA) content; others were used for the determination of mitochondrial membrane potential (MMP), cell cycle and Bcl-2, Bax, CytC protein expression. WBC and PLT numbers in median and high dose groups were obvious reduced, but there was no change on RBC numbers. There was also reduced numbers of bone marrow karyotes and CFU-F in the high dose group. SOD activity was decreased, but MDA content was increased. MMP and Bcl-2 expression were decreased with increasing formaldehyde concentration, while expression of Bax and Cyt C was increased. We also observed change in cell cycling, and found that there was S phase arrest in the high dose group. Our study suggested that a certain concentration of formaldehyde could have toxic effects on the hematopoietic system, with oxidative stress as a critical effect.

**Keywords:** Formaldehyde - SOD - MDA - MMP - cell cycle

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### Introduction

Formaldehyde (FA) is an essential chemical pollutant that is widely used in environmental and industrial activities. Given its economic importance and widespread use, many people are exposed to FA and mainly by inhalation (Zhang et al., 2013). Inhaled FA has been associated with various toxic effects, such as hepatotoxicity, neurotoxicity, reproductive toxicity, respiratory toxicity, et al. Epidemiological survey and experimental data show that occupational and environmental exposures to FA may be associated with an increased risk of leukemia in exposed individuals. The International Agency for Research on Cancer (IARC Monographs, 2012) and the U.S. National Toxicology Program (NTP, 2011) both classified FA as a human leukemogen, but the exact mechanism is not yet clear. As we known, bone marrow (BM) is the site of origination of all blood cells from hematopoietic stem cell (HSC), and it is also the target site for leukemia induction (Renstrom et al., 2010). Nevertheless, there are limited studies on BM toxicity induced by FA.

Increasing evidence has shown that oxidative damage is one of the most critical effects of FA exposure (Zhou

et al., 2006). Oxidative stress is thought to underlie carcinogenesis in humans induced by chemical exposure (Klaunig et al., 2011; Kryston et al., 2011) and is likely to be a main mechanism of leukemogenesis. It is the process of cellular injury caused by excessive levels of reactive oxygen species (ROS), resulting from an imbalance between pro-oxidant and anti-oxidant systems, in order that the excess ROS cause toxic effects and ultimately lead to cell death (Park et al., 2012; Akan et al., 2013; Kong et al., 2013). Studies found that inhaled FA could induce oxidative stress in mouse brain, lung and liver (Matsuoka et al., 2010). However, it has not been clarified about toxic mechanisms of BM induced by inhaled FA. Therefore, the present study was undertaken to explore the possible toxic mechanisms of BM induced by inhaled FA.

### Materials and Methods

Formaldehyde (FA, 36.5-38% in the water, FW 30.03) was obtained from a chemical company in Beijing. SOD and MDA Assay Kits were purchased from Jiancheng Biotechnology (Jiancheng, Nanjing, China). The Bcl-2 and Bax antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, USA). The ethidium iodide,

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RNA enzymes and Rhodamine123 were purchased from Sigma Biotechnology (Sigma, USA). All reagents were of the highest purity commercially available.

#### Animals and treatment

Male ICR mice with body weight of  $13 \pm 1$ g were purchased from the Department of Experimental Animals, Norman Bethune College of Medicine, JiLin University, China (The number for the certificate of animals is SCXK-Ji 2007-0003), and maintained under standard housing conditions (Temperature:  $20 \sim 24^\circ\text{C}$ ; Relative Humidity:  $40 \sim 70\%$ ; Light and Dark Cycle: 12h:12h). Food and water were provided ad libitum. In accordance with the principle of randomization, the mice were divided into four groups and 6 mice per group. One group was exposed to ambient air and the other three groups were exposed to different concentrations of formaldehyde ( $20, 40, 80 \text{ mg/m}^3$ ) for 15 days in the respective inhalation chambers, 2h a day. All animal experiments were treated according to the protocols evaluated and approved by the ethical committee of Norman Bethune Department of Medicine, JiLin University. At the end of the 15-day experimental period, all mice were killed. Bone marrow cells were obtained. Some were used for the determination of blood cells numbers, bone marrow karyote numbers, CFU-F, superoxide dismutase (SOD) activity and malondialdehyde (MDA) content; others were used for determination of mitochondrial membrane potential (MMP), cell cycle and Bcl-2, Bax, CytC protein expression.

#### Determination of blood cells numbers

After FA exposure, the numbers of WBC, RBC and PLT were counted by using a Blood Cell Analyzer (MTN-21, Motenu, Changchun, Jilin, China).

#### Determination of bone marrow karyote numbers and CFU-F

After the mice were killed, put them in 75% ethanol for 5 min, then taken out of the femurs and removed muscles and connective tissues around in a sterile condition. The two ends of the femurs were cut, and the bone marrow cells were washed out by a 1 mL syringe with 1640 cell culture fluid. The bone marrow cells were filtered by a membrane (200 mesh nylon nets) to get a single bone marrow cell suspension. Bone marrow karyote numbers were counted by a blood count plate after the single bone marrow cell suspension was diluted by WBC dilution.

After the single bone marrow cell suspension was made, it was inoculated in culture bottles where the final concentration of nucleated cells was about  $1 \times 10^6/\text{mL}$ . After incubating them for a week at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  incubator, fixed with methanol, stained by Giemsa, and dried in the air. Then observed the cells under the inverted microscope, counted more than 50 cells as a colony.

#### Determination of SOD activity and MDA content

The femurs were taken out, and removed muscles and connective tissues around. The two ends of the femurs were cut, and the bone marrow cells were washed out by a 1 mL syringe with 0.01 mol/L PBS fluid. The bone marrow cells were filtered by a membrane (200 mesh nylon nets)

to get a single bone marrow cell suspension. SOD activity and MDA content were detected by commercial Assay Kits (Jiancheng, Nanjing, China).

#### Determination of MMP

After the single bone marrow cell suspension was made, it was added an equal volume of  $20 \mu\text{g/mL}$  of Rhodamine123 to make a final concentration of  $10 \mu\text{g/mL}$ . Then the suspension was incubated for 30 min at  $37^\circ\text{C}$  in the darkness and washed with PBS fluid, centrifuged for 10 min at 1500 rpm, then repeated again. The cells were detected by FCM after mixed with a tiny 0.01 mol/L PBS fluid.

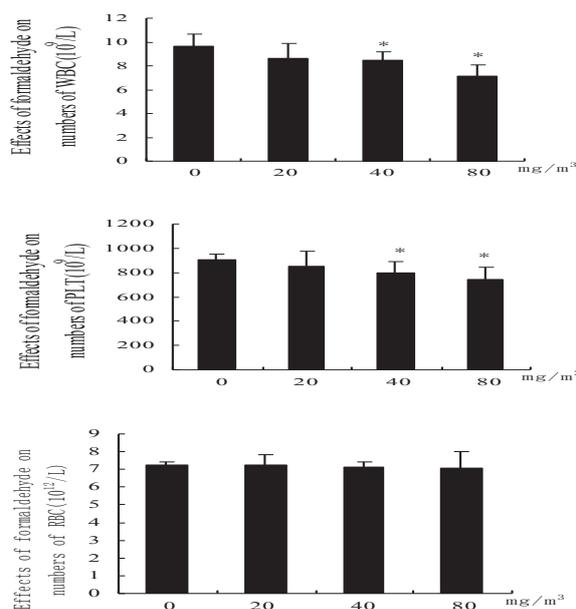
#### Determination of cell cycle

After the single bone marrow cell suspension was made, centrifuged at 1000 rpm for 5 min, then discarded supernatant, washed 2 times with 0.01 mol/L PBS fluid, discarded a most of supernatant. Added precooled 75 % alcohol, and stayed overnight in a refrigerator at  $4^\circ\text{C}$ . The cells was washed 2 times with 0.01 mol/L PBS fluid, and was incubated for 30 min at  $4^\circ\text{C}$  in the darkness after added 100  $\mu\text{L}$  Rnase ( $10 \mu\text{g/mL}$ ) and 100  $\mu\text{L}$  PI ( $5 \mu\text{g/mL}$ ). About  $1 \times 10^4$  cells were collected by FCM and analyzed by Cellquest software.

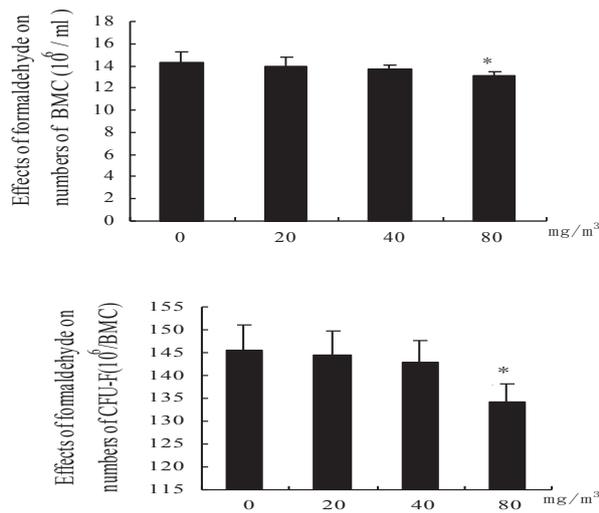
#### Determination of CytC Bcl-2 and Bax Protein Expression by Immunohistochemistry

First we took out the sternums of mice after orbital blood, washed out the bone marrow and conventionally smeared the bone marrow on slides. Then dried slides naturally and fixed them with formalin buffer solution 10% for 15 min, and placed in a refrigerator at  $-20^\circ\text{C}$  after dried. After that, the expression of CytC, Bcl-2 and Bax protein was detected by immunohistochemistry. Finally, the bone marrow cells were observed under the microscope, about 500, and were calculated by the formula:

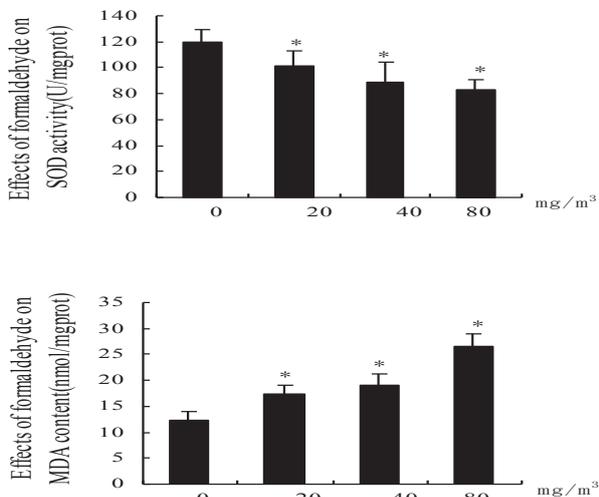
$$\text{Positive cells rate (\%)} = \frac{\text{positive cells}}{\text{cells}} \times 100\%$$



**Figure 1. Effects of FA on Numbers of WBC, PLT and RBC in Mice.** Data are mean $\pm$ SE, \* $p < 0.05$ , compared to control



**Figure 2. Effects of FA on Bone Marrow Karyote Numbers and CFU-F in Mice.** Data are mean±SE, \**p*<0.05, compared to control



**Figure 3. Effects of FA on SOD Activity and MDA Content of Bone Marrow Cells in Mice.** Data are mean±SE, \**p*<0.05, compared to control

**Table 1. Effects of Formaldehyde on Cell Cycle in Bone marrow of mice (n=4,  $\bar{x}\pm s$ , %)**

Group	Cell Cycle		
	G0/G1	S	G2/M
0 mg/m <sup>3</sup>	68.79±2.46	20.73±1.22	10.48±1.94
20 mg/m <sup>3</sup>	67.90±2.45	22.53±2.15	9.57±0.39
40 mg/m <sup>3</sup>	66.43±2.80	25.00±3.37	8.57±0.72
80 mg/m <sup>3</sup>	66.77±1.10	25.60±1.02*	7.63±1.95

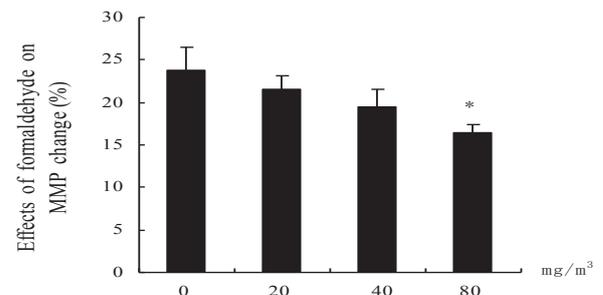
#### Statistical methods

Statistical analysis was performed by SPSS 14.0 statistical software. The difference between the control and formaldehyde-treated groups was tested by one-Way ANOVA analysis. All values are shown as the mean±standard error (SE). The results were considered significant at *p*<0.05.

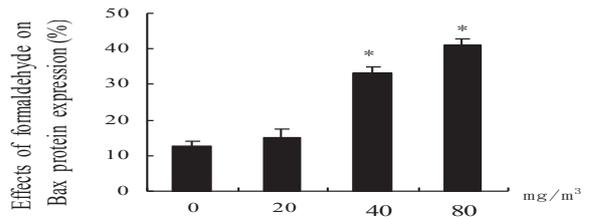
#### Results

##### Effects of FA on WBC RBC and PLT numbers

After exposure to FA, there was an obvious reduced on WBC and PLT numbers in 40 and 80 mg/m<sup>3</sup> formaldehyde-



**Figure 4. Effects of FA on MMP Change of Bone Marrow Cells.** Data are mean±SE, \**p*<0.05, compared to control



**Figure 5. Effects of FA on Bax, CytC, Bcl-2 Protein Expression.** Data are mean±SE, \**p*<0.05, compared to control

treated groups compare to control (*p*<0.05), but there was no change on RBC numbers (Figure 1).

##### Effects of FA on bone marrow karyote numbers and CFU-F

After exposure to FA, numbers of bone marrow karyote and CFU-F in the 80 mg/m<sup>3</sup> formaldehyde-treated group were found to be significantly increased compared to control (*p*<0.05) (Figure 2).

##### Effects of FA on SOD Activity and MDA content

After exposure to FA, SOD activity in each formaldehyde group were found to be significantly decreased compared to control (*p*<0.05), while MDA content was increased compare to control (*p*<0.05) (Figure 3).

##### Effects of FA on MMP

After exposure to FA, MMP was decreased with formaldehyde concentration increasing, and there was an obvious difference in the 80 mg/m<sup>3</sup> formaldehyde-treated group compared to control (*p*<0.05) (Figure 4).

### Effects of FA on cell cycle

After exposure to FA, there was found to cause S arrested in the 80 mg/m<sup>3</sup> formaldehyde-treated group compared to control ( $p<0.05$ ) (Table 1).

### Effects of FA on Bax, CytC and Bcl-2 protein expressions

Bax, CytC and Bcl-2 protein expressions were detected by immunohistochemistry. Results demonstrated that the expression of Bax, CytC protein was obviously increased with the formaldehyde concentration increasing, while the expression of Bcl-2 was decreased. There were obvious differences in 40 and 80 mg/m<sup>3</sup> formaldehyde-treated groups compared to control ( $p<0.05$ ) (Figure 5)

## Discussion

As a highly reactive chemical, FA can cause multiple toxic effects in humans and animals, and it has been classified as a human leukemogen by IARC (IARC Monographs., 2012) and U.S. National Toxicology Program (NTP., 2011), but the exact mechanism is not yet clear, especially there are limited studies on toxic mechanisms in BM, the site of leukemia induction. Blood cells origin from hematopoietic stem cells differentiation in BM, and they have higher sensitivity to chemicals or other environmental factors. So it is possible that the blood system is more susceptible to damage than body's other organs, and one of the clinical consequences of damage is an alternation in RBC, WBC and PLT counts (Zhang et al., 2013). In the present study, we found that there was an obvious reduced on WBC and PLT numbers in median and high dose groups ( $p<0.05$ ), but there was no change on RBC numbers. This result indirectly suggested that FA could induce BM toxicity.

Bone marrow hematopoietic microenvironment is to support and regulate hematopoiesis cells settled, proliferation, differentiation, development, maturity, et al. It can be divided into the stromal cells and the extracellular matrix. The stromal cells are the core of bone marrow hematopoietic microenvironment, and closely contact with hematopoietic cells, so its structural and functional integrity plays an important role. CFU - F can indirectly reflect the situation of bone marrow hematopoietic microenvironment in vivo. In the present study, we found that numbers of bone marrow karyote and CFU-F were increased with FA concentration increasing, and there was an obvious difference in the high dose group compared to control ( $p<0.05$ ). This result demonstrated that FA could cause bone marrow hematopoietic microenvironment damage and induce BM toxicity.

Since hematopoietic system diseases involved in oxidative stress, we hypothesized that it would play a role in FA-induced BM toxicity. ROS, including superoxide anion, hydrogen peroxide, hydroxyl radical, et al. ROS can cause mitochondrial membrane potential (MMP) unstable and mitochondrial dysfunction. In addition, it also can decrease the expression of Bcl-2 protein which can maintain mitochondrial homeostasis and prevent mitochondrial release of Cyt C (Ola et al., 2011). The changes of MMP affect the cell signal transduction, cell cycle regulation and DNA replication, while Cell

cycle checkpoint controls DNA replication and mitotic starting, in order that it can guarantee the completeness and correctness of chromosomes (Wong et al., 2012). Our results demonstrated that SOD activity in each formaldehyde group was found to be significantly decreased, while MDA content was increased. After FA entering the body, it can damage biological membrane and cause bone marrow cells lipid peroxidation, so MDA, as a peroxidation products, its content was increased; in addition, SOD is being consumption in order to defense free radical damage, so SOD activity was decreased. When the body's antioxidant capacity depletion, the generation and elimination of free radicals are out of balance, the oxidative damage of the body will occur. We also detected the changes of MMP and cell cycle by FCM, the expression of Bcl-2, Bax and Cyt C protein. Results demonstrated that MMP and Bcl-2 protein expression were decreased with formaldehyde concentration increasing, while the expression of Bax and Cyt C protein was increased. We also observed that there was S arrested in FA-induced BM toxicity. If the cells were stuck at S-phase, too much DNA in these cells couldn't enter into M-phase, so as to cause cell division and abnormal proliferation, affect the normal cell cycle progression. Once the damaged cells can't be repaired, they will enter into apoptosis.

In conclusion, the present results demonstrated that FA can induce adverse effects on BM, which might increase the risk of hematopoietic system diseases. Oxidative stress may be an underlying mechanism during this process.

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