## Letter to the Editor

# Dimethylacetamide-induced Hepatic Injury *in Vitro*: Mechanism and Potential Preventive Strategy*

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***N,N*-Dimethylacetamide (DMAc) is a widely used organic solvent in modern chemical industry with low to moderate hepatotoxicity to occupational health of employees. But so far, there are fewer and less conclusive data concerning its pathogenic mechanism in detail. In current study, the toxicity of DMAc was firstly investigated on human normal hepatocytes (LO-2), using a series of molecular biology measurements to ananlyze the effect and mechanism of DMAc-induced hepatic cell injury and explore effective prophylactic measures. We found that DMAc triggered LO-2 apoptosis in a obviously dose-dependent manner, caused by increased ROS generation and activation of Bcl-2 pathway. Significantly, glutathione (GSH) rather than vitamin C (Vit C) could partially inhibit DMAc-induced apoptosis thus showing potential as a effective precaution for workers.**

DMAc (CAS No. 127-19-5) is a colorless liquid with a faint ammonia-like odor. It is widely used as a solvent for many organic reactions in the manufacture of synthetic fibre and resins, as well as the production of adhesives, plasticizers, and pharmaceuticals[1]. With relatively low vapor pressure, contact with both the dermal and respiratory systems is the main source of human exposure. Despite occupational exposure limits and industrial hygiene practices to limit dermal contact, DMAc use has been associated with occupational illness, especially in Asia where new and expanded uses have led to over exposures[2]. The liver is the mainly involved target organ of DMAc. The American Conference of Governmental Industrial Hygienists (ACGIH) has already proposed the threshold limit value (TLV) to minimize hepatic injury and jaundice.

The hepatotoxicity of DMAc is well validated in animals. Fatty infiltration, increased liver weight, biliary hyperplasia, hepatocellular degeneration and necrosis have been revealed in several animal models[3]. Data on human hepatic injury due to DMAc exposure has also been published. For example, Baum and Suruda published two case studies of DMAc-related toxic hepatitis in employees working on an acrylic-fiber production line 2 weeks and 3 months after first contact[4]. Seven cases of DMAc-induced hepatitis associated with environmental exposure at an elastane fibre plant was reported by Jung et al.[2]. Epidemiological surveys suggest a dose-response relationship between occupational exposure to DMAc and liver damage. Kim et al. found 34 cases of hepatic injury relating to DMAc among elastane fibre workers over 16 months of occupational disease surveillance[5]. Our preliminary investigation of occupational exposures in spandex and electronic production factories found that a higher proportion of workers experienced liver dysfunction, including acute hepatitis, after vocational contact with DMAc.

Although the health effects from occupational exposure to DMAc are well known, there are fewer and less conclusive data concerning its pathogenic mechanism. This study examines the toxicity of DMAc on human normal hepatocyte (LO-2) using a series of molecular biology methods. Our objectives were to analyze the effect and mechanism of DMAc-induced hepatic cell injury and explore effective prophylactic measures, such as using antioxidants, to improve the health of DMAc-exposed workers.

Human liver cell line (LO-2) obtained from the American Type Culture Collection (ATCC) was maintained at 37 °C in RPMI-1640 medium supplemented with 10% v/v fetal bovine serum, penicillin (100 μg/mL) and streptomycin (100 μg/mL). Cells were then incubated with DMAc (Sigma, USA) for 24 h to mimic hepatic injury following exposure. N-acetyl cysteine (NAC, Sigma, USA) was used to scavenge ROS, and L-Glutathione (L-GSH, Sigma,

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USA), Sodium L-ascorbate (Vit C, Sigma, USA) were applied as antioxidants 24 h prior to DMAc treatment.

We evaluated cell viability by the CCK-8 test (Cell Counting Kit-8, Takara, Japan). Cells were placed on a 96-well culture plate at a density of $2\times10^3$/well in 0.1 mL of culture medium. After incubation for 12 h, the wells were divided into six groups receiving different levels of DMAc. At the end of the specified incubation period, images were taken with a Nikon inverted phase contrast microscope (Nikon, Japan) (200 ×) equipped with the Quick Imaging system for cell morphological analysis. Then 10 μL CCK-8 reagent was added to each well. After 4 h, absorbance was measured optical density (OD) at 450 nm with a multidetection micro plate reader (MD, USA).

Apoptotic cells were detected by Hoechst 33258 staining following the manufacturer's protocol (Hoechst staining kit, Beyotime, China). We examined and photographed the stained cells under a fluorescence microscope (Olympus, Japan). In each group, six microscopic fields were selected randomly and counted.

Intracellular ROS was detected by means of an oxidation-sensitive fluorescent probe 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA) according to the manufacturer's instructions (ROS assay kit, Beyotime, China). The green fluorescence was observed and immediately photographed. In each group, six microscopic fields were selected randomly and the number of cells marked with green fluorescence was counted *via* Image J software (NIH, USA), respectively.

Fluorescence recovery after photobleaching (FRAP) assay was based on previously published methods. The FRAP reagent was prepared according to the instruction (T-AOC assay kit, Beyotime, China). The standard curve was constructed using iron (II) sulfate solution, and the results were expressed as nmol/L $FeSO_4$.

Intracellular content of GSH was measured as described by instruction supplied with the GSH and GSSG Assay Kit (Beyotime, China). Standards of GSH and a sample blank lacking GSH were also assayed at the same time.

Protein expression level was determined by western blotting. Transferred blots were incubated sequentially with 5% non-fat milk, primary antibodies against p53, Bcl-2, Bax, cleaved caspase-3, cleaved caspase-9, actin and HRP conjugated secondary antibodies (CST, USA). Protein bands were visualized with an enhanced chemiluminescence detection kit (Beyotime, China) and recorded on gel imaging system (Bio-rad, USA).

Cell culture experiments were repeated at least three times with six replicates at each concentration. All calculations and statistical analysis were performed using SPSS for Windows version 20.0. One-way analysis of variance (ANOVA) was used to analyze the difference between groups. $P<0.05$ was regarded as statistically significant.

After confirming the concentration and exposure time of DMAc, viability of LO-2 treated for 24 h was assessed by CCK-8 assay. Cell viability was significantly inhibited at concentrations beyond 25 mmol/L. The treatment of LO-2 with 25-125 mmol/L of DMAc resulted in a dose-dependent inhibition of cell growth compared with control (Figure 1A). Meanwhile, cellular morphology clearly altered under DMAc irritation. As shown in Figure 1B, normal LO-2 cells were oval or diamond-shaped. However, when treated with DMAc, cell shape became irregular gradually, exhibiting retraction, rounded and reduced size with the increase of DMAc dosage.

The changes in nuclear morphology of apoptotic cells were examined by Hoechst 33258 staining. The normal nucleus showed a homogeneous staining, bearing regular contours and rounded shapes. Apoptotic cells showed an asymmetrical, highly bright fluorescence, and the number of condensed nuclei increased after exposed to DMAc for 24 h (Figure 1C). The ratio of apoptotic cells obviously increased along with the concentration of DMAc (Figure 1D).

ROS generation was evaluated using intracellular peroxide-dependent oxidation of DCFH-DA to form fluorescent DCF (Figure 2A). ROS production was markedly elevated upon treatment with 25-125 mmol/L DMAc compared to control (Figure 2B). Total antioxidant capacity was significantly decreased in the meantime (Figure S1A, www.besjournal.com for details). NAC is a kind of specific ROS scavenger. Pretreatment with 1-5 mmol/L NAC effectively attenuated DMAc-induced LO-2 growth inhibition (Figure S1B, www.besjournal.com for details), meaning DMAc-triggered apoptosis was dependent on ROS induction.

L-GSH and Vit C are classic antioxidants that can protect cells against oxidative stress injury. We wanted to study whether these antioxidants could protect LO-2 from DMAc-induced apoptosis. As

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