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The toxic effects of melamine on spleen lymphocytes with or without cyanuric acid in mice

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ABSTRACT

Melamine is an organic nitrogenous compound whose acute toxicity was generally thought to be low in animals. In the present work, we investigated the potential cytotoxic effects of melamine on spleen lymphocytes in mice. In the treated group, morphological changes were observed in cultured lymphocytes in vitro. The co-administration of melamine and cyanuric acid caused a declining tendency in stimulation index of spleen lymphocyte. All treated groups had lower ratios of CD4+/CD8+. Both early apoptotic and late apoptotic/necrotic rates of lymphocyte were significantly higher in the co-administration high groups of melamine and cyanuric acid. Melamine-related toxicity promoted the expression of Bax mRNA, and suppressed the expression of Bcl-2 mRNA in spleen of the treated mice. These results provided useful information for assessing the toxicity of melamine on immune system of mammals, and contributed to the existing toxic profile of melamine

1. Introduction

Melamine (2,4,6-triamino-1,3,5-triazine, $C_3H_6N_6$), a synthetically produced chemical, is commonly used in the production of plastics, laminates, glues, fertilizers, kitchenware, adhesives and other products (Sun et al., 2010; Wu et al., 2013). In previous investigations, it was reported that the acute toxicity of melamine alone was generally thought to be low in animals (Brown et al., 2007; Melnick et al., 1984). In the 1960s and 1970s, it was recorded that melamine was also explored as potential anti-cancer agent, but was afterwards discarded considering its lack of efficacy (Langman et al., 2009). Since 2008, melamine has attracted great social attention in that it was found to be illicitly added to infant formulas to falsely increase the protein content (Puschner and Reimschuessel, 2011), and caused serious health problem for many Chinese children (World Health Organization, 2008; Guan et al., 2009). In recent years, there has been growing concern about kidney failure resulting from melamine-related toxicity (Hau et al., 2009; Yin et al., 2013a). It is well known that the combination of melamine and cyanuric acid (1,3,5-triazine-2,4,6-triol, $C_3H_3N_3O_3$) led to the formation of almost insoluble crystals, and subsequent renal impairment in animals (Pang et al., 2013; Reimschuessel et al., 2008).

As shown in increasing numbers of investigations, however, the melamine-related toxicity might not be limited to kidney (Hau et al., 2009; Yin et al., 2013a; Yoon et al., 2011). In a recent investigation, we also showed that melamine can cause ultrastructural pathological injury to the liver, kidney, spleen, stomach wall, and small intestine of mice in a dose-dependent pattern, and the combination of melamine and cyanuric acid (each at 25 mg/kg/day for 14 consecutive days) has been shown to be more toxic to the examined organs than melamine alone (50 mg/kg/day for 14 consecutive days) (Yin et al., 2013b). Moreover, it was reported that melamine not only can cause sperm deformity (Yin et al., 2013a; Zhang et al., 2011), but also inhibit the proliferation of differentiated PC12 cells (Han et al., 2011), as well as, affect the morphology of hippocampus neurons (Wang et al., 2011). These findings imply that melamine with or without cyanuric acid appear to be systemically cytotoxic to many different cell types in animals (Yin et al., 2013b).

It is well known that many of the cell lines used in studies in vitro might lose some of their original characteristics modulating their response in toxicological testing. Lymphocytes are of fundamental importance in the immune system of the body. They do not contain enzymes that produce the toxic metabolite form of the parent drug, but contain the enzymes that are required for detoxification, thus, once there are any genetic deficiencies in these

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enzymes, the defects would be phenotypically expressed in lymphocytes (Shear and Spielberg, 1988). In many previous studies, lymphocytes were successfully used as a surrogate cell type in testing the potential genotoxicity of different bioactive compounds with protective properties (Dhawan et al., 2002; Jagetia et al., 2003; Lee et al., 2004; Scarpato et al., 1998; Yamakage et al., 1998). More recently, lymphocytes were also used as a model system to evaluate the cyto/genotoxicity of oxime K048 in humans (Vrdoljak et al., 2014).

To our knowledge, however, little information could be found that have determined the toxic effects of melamine on spleen lymphocytes with or without cyanuric acid in mammals. Thus, the purpose of the present work was to investigate the potential cytotoxic effects of melamine on spleen lymphocytes in the absence and presence of cyanuric acid in terms of morphology, proliferation and apoptosis of lymphocyte in mice.

2. Materials and methods

2.1. Animals

A total of 56 male healthy mice of the Kunming strain weighing 20 ± 2 g were provided by Liaoning Changsheng Biological Technology Co., Ltd. [license No.: SCXK (Liao) 2010-0001, Benxi, Liaoning, China]. All animal protocols were reviewed and approved by the Animal Experimental Committee of Shenyang Agricultural University.

2.2. Animal grouping and administration for experiments in vivo

After 7 days of acclimation, the mice were randomly divided into seven groups (each group, $N = 8$), including one control group, three melamine group (low, middle, and high doses) and three mixture group of melamine and cyanuric acid (low, middle, and high doses). The animals were maintained in controlled laboratory conditions of 12 h dark/light cycle, 22 ± 2 °C temperature, and 35–65% relative humidity. The mice had free access to water and standard laboratory food (containing 24% protein, 4% fat and about 5% fiber) provided by experimental animal center of Liaoning University of Traditional Chinese Medicine (Shenyang, China). All feeds and water were subjected to the detection for possible contaminant of both melamine and cyanuric acid as described method by Heller and Nochetto (2008). Neither melamine nor cyanuric acid contaminant was detected in the feeds and water used for this study above the limit of 0.5 ppm.

The mice of control group were administered 1 mL of physiological saline. The mice of melamine group were administered with melamine (>99%, Sinopharm Chemical Reagent Beijing Co., Ltd, Beijing, China) at doses of 2 mg/kg (low dose), 10 mg/kg (middle dose) and 50 mg/kg (high dose) once every 2 days. The mice of mixture groups were administered with the combination of melamine and cyanuric acid (>98%, Shanghai Crystal Pure Industrial Co., Ltd, Shanghai, China) at the dosages consisting of 1 mg/kg (low dose), 5 mg/kg (middle dose) and 25 mg/kg (high dose) of each once every 2 days. We carried out the all administrations via gastric gavages for 30 days. At the end of the experiment, all mice of each group were sacrificed by cervical dislocation, and the spleens were collected immediately from each mouse.

2.3. Morphological analysis of cultured lymphocytes in vitro

Lymphocytes were isolated from the spleen of healthy mice using the standard methodology. The viability of lymphocyte was assessed with trypan blue dye exclusion being more than 95%. The lymphocytes were diluted to 1×10^6 cells/mL with RPMI-1640 broth, and then were treated in a 96 well microplate following the assay listed in Table 1. Lastly, the cells were maintained at 37 °C with 5% CO_2 humidified atmosphere. Untreated lymphocytes were considered as controls. After being cultured for 24 h, morphological changes of lymphocytes were analyzed using inverted phase contrast microscope (Biozero BZ8000, Keyence, Osaka, Japan).

Table 1
Grouping and treatment of mouse lymphocytes cultured in vitro.

Number	Groups	Replications	Concentrations of melamine (mg/L·day)	Concentrations of cyanuric acid (mg/L·day)
1	Control	6	0	0
2	Melamine	6	0.25	0
3	Melamine	6	2.5	0
4	Melamine	6	25	0
5	Melamine	6	250	0
6	Combination of melamine and cyanuric acid	6	0.125	0.125
7	Combination of melamine and cyanuric acid	6	1.25	1.25
8	Combination of melamine and cyanuric acid	6	12.5	12.5
9	Combination of melamine and cyanuric acid	6	125	125

2.4. Analysis of proliferation and subsets of lymphocytes

Lymphocytes were isolated from the spleen of treated mice according to the method described by Cox et al. (2002). The cell suspension of 100 μL was added to each well in a 96 well microplate, and maintained at 37 °C for 44 h in a humidified atmosphere with 5% CO_2. Each test was carried out in triplicate. Using MTT assay described by Xue et al. (2012), the optical density (OD) values at 570 nm were measured for analyzing the proliferation of lymphocytes. The Stimulation index (SI) was calculated the ratio of OD value of the test group against control group.

The treated lymphocyte suspension was diluted to 1×10^7 cells/mL. Aliquots of cell suspension were incubated with a mixture of the anti-CD3-FITC and anti-CD4-PE (5 μL each) or anti-CD3-FITC and anti-CD8-PE (5 μL each) antibodies at 4 °C for 30 min in a dark environment. The cells were fixed with 1% paraformaldehyde solution, and analyzed by flow cytometry. About 10, 000 lymphoid cells were examined for each sample.

2.5. Detection of lymphocyte apoptosis in mice

The lymphocyte suspension from the treated mice with different drugs (2, 10 and 50 mg/kg/day, respectively) was stained separately with annexin V-FITC and propidium iodide for 10 min in a dark environment. The detection of cell apoptosis was carried out by flow cytometry.

2.6. Expression detection of apoptosis-related genes Caspase-3, Bax and Bc1-2 in the spleen of treated mice

The total RNA was extracted from approximately 100 mg spleen tissue of each mouse using the RNAiso reagent kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. The integrity of total RNA was verified by formaldehyde denaturing agarose gel (1%, w/v) with ethidium bromide staining. Using ultraviolet spectrometer, the purity and quantity of the total RNA were assessed with the ratio of OD_{260}/OD_{280} being 1.8–2.0 for all the samples. The total RNA isolated was treated with DNase I (TaKaRa) to exclude the residual genomic DNA. Using M-MuLV cDNA Synthesis Kit (Sangon,