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The effects of melamine on humoral immunity with or without cyanuric acid in mice

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ABSTRACT

Melamine is an industrial chemical with high nitrogen content. When added to the pet food and milk it can falsely elevate the apparent protein concentration readings. Cyanuric acid related structurally to melamine has a strong mutual affinity with melamine. The combined ingestion of melamine and cyanuric acid was considered to be responsible for the crystalluria, kidney stones and subsequent renal failure in animals. In our previous investigation, we demonstrated that melamine alone or its combination with cyanuric acid appears to be toxic to the immune system in mice. The objective of this study was to investigate the potential effects of melamine on humoral immunity with or without cyanuric acid in mice. In comparison to control group, a significantly lower content of plasma cells expressing CD138 were observed in mixture groups of melamine and cyanuric acid with both middle and high doses. The co-administration of melamine and cyanuric acid resulted in a significant decreasing in blimp-1 protein expression and the contents of sIgA, C3, IL-21 and IL-4 compared with the control group. Moreover, our data clearly showed that melamine-related toxicity suppressed the production of IL-6 and IL-10 in a dose-dependent manner. Also, the animals from mixture of melamine and cyanuric acid with high dose group exhibited a significantly lower expression of gata-3 protein, The results from the present study suggested that the exposure to melamine alone or combination with cyanuric acid had certain humoral immunotoxicity in mice, especially when ingested in high dosage.

1. Introduction

Melamine is an industrial chemical with high nitrogen content (approximately 66% by molecular weight). In previous studies, it was indicated that melamine by itself might be very low acute toxicity with an oral LD_{50} of 3161 mg kg^{-1} d^{-1} in rats and mice (National Toxicology Program, 1983), and greater than 90% of the ingested melamine was eliminated from the body within 24 h (Mast et al., 1983). Since 2008, however, melamine has attracted increasing social attention due to the unfortunate occurrences of renal injury in pets and infants (World Health Organization, 2008; Reimschuessel et al., 2008). It was revealed that the combined ingestion of melamine and cyanuric acid caused the crystalluria, kidney stones and subsequent renal failure (Hau et al., 2009; Chen et al., 2009). Therefore, most researches on the melamine-related toxicity were mainly focused on renal stone formation.

In recent years, however, increasing numbers of studies demonstrated that melamine-related toxicity appears to not be limited to renal injury (Hau et al., 2009; Yin et al., 2013). It was recorded that chronic exposure to melamine may cause reproductive damage with the eye, skin and respiratory irritant (Yoon et al., 2011; Stine et al., 2014). For instance, several investigations demonstrated that melamine had clear testicular toxicity in mice with the increase of sperm abnormality rate and DNA damage, especially when ingested in high concentration (Zhang et al., 2011; Yin et al., 2013). Also, it was reported that both melamine and cyanuric acid could disrupt the blood-testis barrier in mice even at a low-dose level (Chang et al., 2014). The potential toxicity of melamine on nervous system was also investigated by An et al. (2012) demonstrating that the actions of melamine are selective for hippocampus and that melamine could induce the excessive generation of free radicals and lipid peroxidation in the hippocampus, and further affect the oxidation–antioxidation homeostasis. More recently, another investigation by An and Zhang (2014) indicated that prenatal melamine exposure impaired hippocampal synaptic function.

In our recent investigation, we found that the ingestion of melamine alone or combination with cyanuric acid had certain toxic effects on spleen lymphocytes of mice (Yin et al., 2014), suggesting that melamine alone or its combination with cyanuric acid appear to be toxic to the immune system in animals. Humoral immunity is an aspect of immunity

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that is mediated by macromolecules found in extracellular fluids such as secreted antibodies, complement proteins and certain antimicrobial peptides. It plays important roles in protecting the body from infection (Carroll, 2008). To our knowledge, however, there is a dearth of information about the toxic effects of melamine alone or its combination with cyanuric acid on the humoral immunity in animals. Thus, the objective of this study was to investigate the potential effects of melamine on humoral immunity with or without cyanuric acid in mice. Several parameters were detected including plasma CD138, blimp-1, IgG, sIgA, C3, $CD4^+CXCR^+$, IL-21, IL-4, IL-6, IL-10 and gata-3. Overall, those parameters are tightly associated with humoral immunity. For example, the blimp-1 deficiency in mice was demonstrated to result in a severe reduction in the humoral immune response (Shapiro-Shelef et al., 2003; Savitsky and Calame, 2006). The results from the present study provide useful information for evaluating the melamine-related toxicity to immune system in animals, and to supplement the existing toxic profile of melamine.

2. Materials and methods

2.1. Animal grouping and administration

Healthy male mice of the Kunming strain ($N = 56$) weighing 18–22 g were purchased from Liaoning Changsheng Biological Technology Co., Ltd. [license No.: SCXK (Liao) 2010-0001, Benxi, Liaoning, China]. The mice were acclimated for 7 days, and then were assigned randomly to seven groups (each group, $N = 8$). They are one control group, three melamine groups (low, middle, and high doses) and three mixture groups of melamine and cyanuric acid (low, middle, and high doses). The animals were housed in a controlled laboratory conditions maintained at 20–24 °C temperature, 35–65% relative humidity and a 12-h light and dark cycle. Standard laboratory food (experimental animal center of Liaoning University of Traditional Chinese Medicine, Shenyang, China) and water were provided ad libitum throughout the study. The diet was confirmed negative for both melamine and cyanuric acid as described method by Heller and Nochetto (2008). Animal use and procedures were reviewed and approved by the Animal Experimental Committee of Shenyang Agricultural University.

In the melamine groups, the mice were given 2 mg/kg (low dose), 10 mg/kg (middle dose), and 50 mg/kg (high dose) melamine (>99%, Sinopharm Chemical Reagent Beijing Co., Ltd., Beijing, China) once every 2 days. In the mixture groups of melamine and cyanuric acid, the mice were given 1 mg/kg (low dose), 5 mg/kg (middle dose) and 25 mg/kg (high dose) both melamine and cyanuric acid (> 98%, Shanghai Crystal Pure Industrial Co., Ltd., Shanghai, China) once every 2 days. In the control group, the mice were given 1 mL physiological saline. We carried out the all administrations by gastric gavages for 30 days. Subsequently, all experimental animals received 0.1 mL SRBC (1%) by intraperitoneal injection to stimulate the body humoral immune response. After 24 h of intraperitoneal injection with SRBC, the blood was collected from orbital sinus of each mouse, and then we sacrificed the all mice of each group via cervical dislocation. The spleens were collected immediately from each mouse.

2.2. Analysis of plasma cells expressing CD138 and Tfh cell expressing $CD4^+CXCR5^+$

Lymphocytes were isolated from the spleen of each mouse using the method described by Cox et al. (2002), and washed three times in PBS by centrifugation at 2000 rpm for 5 min. Lymphocyte suspension was diluted to 1×10^7 cells/mL. Dead cells were removed by passing through Lympholyte-M (Cedarlane Laboratories, Burlington, NC), and then the remaining cells were incubated with PE anti-mouse CD138 (clone 281-2, 0.2 mg/mL, Biolengend, CA, USA), FITC anti mouse CD4 (clone GK1.5, 0.5 mg/mL, Biolengend, CA, USA) and PE anti mouse CXCR5 (clone L138D7, 0.2 mg/mL, Biolengend, CA, USA) at 4 °C for 20 min in a dark environment, respectively. A protocol without any anti-body was used as control. Subsequently, the cells were washed for three times in PBS-buffer (pH 7.3) and were fixed with fresh 4% paraformaldehyde solution. Lastly, the cells were analyzed on a FACScan machine (BD Biosciences, CA, USA). Approximately 10, 000 cells were examined for each sample.

2.3. Analysis of blimp-1 protein and gata-3 gene expression and the contents of IgG, sIgA and complement C3

To demonstrate potential expression changes of blimp-1 and gata-3 proteins, Western blotting was performed as the method described by Posadas et al. (2010). Briefly, spleen tissues samples of each mouse were homogenized in RIPA buffer, and the total protein was isolated using RNA–DNA-protein separation reagent (Progen Industries, Brisbane, Australia). Using Bradford reagent (Sigma, MO, USA), the protein concentration was quantified with BSA as a standard. The samples was separated on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto PVDF membrane. After blocking using 5% non-fat dry milk in PBS for 2 h, membrane was incubated overnight using purified mouse anti-blimp-1 and mouse anti-gata-3 antibodies (Biolengend, CA, USA) at a dilution of 1:1000, respectively. Horseradish peroxidase-conjugated goat anti-mouse IgG (Biolengend, CA, USA) at a dilution of 1:7000 was used as a secondary antibody. Finally, on X-ray film (Bio-Rad, CA, USA), the signals were detected using a Chemiluminescence detection system (Pierce, IL, USA). Anti-β-actin antibody (Sigma, MO, USA) was used as an internal control.

The real-time quantitative RT-PCR (qPCR) was used to demonstrate potential mRNA expression change of gata-3 gene. Total RNA was extracted from about 100 mg spleen tissues with the RNAiso RNAiso Plus (TaKaRa, Dalian, China) following the manufacturer's protocol. The integrity of total RNA was verified by analyzing approximately 1 μg RNA sample on 1% (w/v) formaldehyde denaturing agarose gel. The quantity and purity of extracted RNA were assessed on an ultraviolet spectrometer. The 260/280 ratio was verified to be between 1.8 and 2.0, and the 260/230 ratio was greater than 1.6 in all analyzed samples. The DNase I (RNase-free, TaKaRa, Dalian, China) was used in order to remove residual traces of genomic DNA. The first strand cDNA was synthesized with the PrimeScript TM RT reagent Kit (TaKaRa, Dalian, China) following the manufacturer's direction. A protocol without template RNA was used for each sample as a negative control. The expression level of Gata-3 gene was measured with SYBR Premix Ex Taq (TaKaRa, Dalian, China). The β-actin gene was used for qPCR data normalization based on the previous investigation by Bao et al. (2010).

Primer sequences were as follows: Gata-3-F: 5′-tggtaccgggcactacctttg -3′ and gata-3-R: 5′- atcctgcgcgaactgtcagac -3′; and β-actin -F: 5′- ctgtccctgtatgcctctg -3′ and β-actin -R: 5′-ttgatgtcacgcacgatt -3′. On an ABI 7300 real-time PCR system (Applied Biosystems, CA, USA), the qPCR reaction was carried out in a 15 μL final volume containing: 7.5 μL SYBR Premix Ex Taq (2× concentration) (TaKaRa, Dalian, China), 0.3 μL ROX Reference Dye (50× concentration), 2.25 μL forward and reverse primers (2 μM), 2.0 μL first-strand cDNA, and 2.95 μL PCR grade water. The following cycling parameters were used for the qPCR reaction: 95 °C for 2 min, then 40 cycles of the following: 95 °C for 15 s, 60 °C for 10 s, and 72 °C for 20 s. Three replicates were included in each sample with a negative control. The dissolve curve analysis was performed by heating from 65 to 95 °C with increases of 0.2 °C per step with the system held 1 s at each temperature. The results were analyzed using the $2^{-\triangle\triangle Ct}$ method as described by Livak and Schmittgen (2001).

To determine the content changes of IgG, sIgA and complement C3, sera were separated from blood samples by centrifugation at 3000 rpm for 20 min at room temperature, and were stored at −80 °C for further use. The concentrations of IgG, sIgA and complement C3 in each sample were determined by commercially available ELISA kits (Shanghai Hufeng Biotechnology Co., Ltd., Shanghai, China). The lower