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The reproductive toxicity of melamine in the absence and presence of cyanuric acid in male mice

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ABSTRACT

Melamine, a chemical compound, was used widely in the manufacture of amino resins and plastics. Cyanuric acid related structurally to melamine was used as a water stabilizer in swimming pools. The combination of melamine and cyanuric acid was thought to be responsible for renal impairment in mammals. In the present work, we investigated the reproductive toxicity of melamine in the absence and presence of cyanuric acid in male mice. Pathological damages in different degrees were observed in the testis of male mice treated with different doses of both melamine alone and combination of melamine and cyanuric acid in a dose-dependent manner. Based on the TUNEL assay, the mice treated with high dose of melamine (50 mg/kg/day) had a significant increase in apoptotic index of spermatogenic cells ($p < 0.05$) compared with the control group. Sperm abnormality test indicated that melamine alone resulted in abnormal sperm morphology. The mice from co-administration groups of melamine and cyanuric acid were not eating, and were most likely in renal failure. The combined exposure to melamine and cyanuric acid was revealed to have certain toxic effects on testis of male mice at a relative low dose (each at 1 mg/kg/day). Also, in comparison to melamine treated groups, more severe apoptosis was observed in co-administration groups of melamine and cyanuric acid with both middle (each at 5 mg/kg/day) and high doses (each at 25 mg/kg/day). However, all mice administrated with combination of melamine and cyanuric acid (each at 206, 412, or 824 mg/kg/day) died before day 6 from which no data were obtained on sperm abnormality. These results from this study demonstrated that melamine had certain toxic effects on testes of male mice, especially when ingested in high concentration. These results might be useful in evaluating the toxicity of melamine on reproductive system of male animal, and they also would be a supplement to the existing toxic profile of melamine.

1. Introduction

Melamine (2,4,6-triamino-1,3,5-triazine), a chemical material, is widely used in manufacturing laminates, plastics, coatings, commercial filters, glues, dishware, and kitchenware (Shen et al., 2011; Xue et al., 2011). Due to its 66% nitrogen by molecular weight, melamine could increase the apparent protein concentration readings (Langman et al., 2009; Puschner and Reimschuessel, 2011). So, melamine was added intentionally to the foodstuffs to elevate falsely the apparent protein content (Pang et al., 2011). Previous studies demonstrated that the acute toxicity of melamine alone was generally low in mammals (Melnick et al., 1984; Brown et al., 2007; Baynes et al., 2008). In 2008, however, approximately 294,000 Chinese children were affected by the infant formula contaminated with melamine, of which 51,900 underwent hospitalization and 6 died from renal failure (World Health Organization, 2008; Guan et al., 2009). Over the last few years, moreover, we have increasingly appreciated that chronic exposure on the melamine may cause reproductive damage or cancer (Yoon et al., 2011).

Cyanuric acid (1,3,5-triazine-2,4,6-triol) related structurally to melamine was used as a water stabilizer in swimming pools and hot tubs to minimize the decomposition of hypochlorous acid by light (Downes et al., 1984). Although, cyanuric acid itself is of rather low acute toxicites, it has a strong mutual affinity with melamine (Pang et al., 2011; Puschner and Reimschuessel, 2011). Studies have demonstrated that the combination of melamine and cyanuric acid caused renal impairment through forming nearly insoluble crystals in different species, such as cat (Puschner et al., 2007), dog (Burns, 2007), pig and fish (Reimschuessel et al., 2008), rat (Pang et al., 2011). Furthermore, the mechanisms of

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renal stone formation and renal failure induced by co-administration of melamine and cyanuric acid were also investigated by Kobayashi et al. (2010). Recently, Xie et al. (2011) reported that combined administration of melamine and cyanuric acid resulted in liver damage exhibiting an obvious dose-effect pattern. However, it has been less extensively investigated on the reproductive toxicity of melamine in the absence and presence of cyanuric acid in male animals (Zhang et al., 2011).

The male reproductive system consists of the testis and other accessory structures responsible for sperm production, which occurs in the seminiferous tubules of the testis. Agents that alter testicular function will affect the quality and quantity of spermatozoa (D'Cruz et al., 2010; Heidari et al., 2012). Compared with other system, the reproductive system is more sensitive to toxic chemicals (Klimowicz et al., 2008; Momose-Sato et al., 2009). To our knowledge, however, little information could be found that have determined the reproductive toxicity of melamine with or without cyanuric acid in male animals. The purpose of the present work was to investigate the potential reproductive toxicity of melamine in the absence and presence of cyanuric acid in male mice in terms of testicular histopathology, apoptosis of spermatogenic cell and sperm morphology.

2. Materials and methods

2.1. Animals

Healthy Kunming male mice (n = 120) weighing 25–30 g were used for all studies. Mice were provided by Beijing Fukang Biological Technology Co., Ltd. (license No.: SCXK 2009–0004, Beijing, China). All animal protocols were reviewed and approved by the Animal Experimental Committee of Shenyang Agricultural University.

2.2. Chemicals and reagents

Melamine (>99%) was obtained from Sinopharm Chemical Reagent Beijing Co., Ltd (Beijing, China). Cyanuric acid (>98%) was purchased from Shanghai Crystal Pure Industrial Co., Ltd (Shanghai, China). In situ apoptosis detection kit was purchased from Roche Molecular Biochemicals (Mannheim, Germany). Proteinase K was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Diamino-benzidine (DAB) staining kit and Polylysine were purchased from Wuhan Boster Biological Technology., Ltd. (Wuhan, China). Ultrapure water was obtained from a Millipore system (Bedford, MA, USA). Other chemicals were analytical grade.

2.3. Animal grouping and administration

All mice were acclimated to the environmental conditions for 15 days prior to the study, and they had free access to water and standard laboratory food (containing 24% protein, 4% fat and about 5% fiber) obtained from experimental animal center of Liaoning University of Traditional Chinese Medicine (Shenyang, China). Prior to use, all feeds and water were analyzed for both melamine and cyanuric acid according to the described method of Heller and Nochetto (2008), and neither melamine nor cyanuric acid contaminant was detected above the limit of quantitation of the method being 0.5 ppm. The animals were maintained in controlled laboratory conditions of 22 ± 2 °C temperature.

To investigate the histopathologic changes and apoptosis of spermatogenic cells of testis in male mice, 56 mice were randomly selected and divided into seven groups (each group, N = 8), including one control group, three melamine groups (low, middle, and high doses), and three Mix groups of melamine and cyanuric acid (low, middle, and high doses). The eight animals of each group were housed together.

The mice of control group were administered 1 mL of physiological saline daily. The mice of melamine group were administered with melamine alone, at doses of 2 (low dose), 10 (middle dose), or 50 (high dose) mg/kg/day. The mice of Mix groups were administered with the combination of melamine and cyanuric acid at the dosages consisting of 1 (low dose), 5 (middle dose), and 25 (high dose) mg/kg/day of each. Melamine and cyanuric acid were mixed with water at room temperature, and each dose was mixed just before dosing each animal. All administrations were made via gastric gavages for 14 consecutive days. At the end of the experiment, all mice of each group were sacrificed by cervical dislocation, and the testis tissues were collected immediately from each mouse. Body weight was monitored on days 3, 8 and 14.

To evaluate the sperm abnormality of male mice, another 64 mice were randomly divided eight groups (each group, N = 8), including one negative control group, three melamine groups, three Mix groups of melamine and cyanuric acid and one positive control group. The eight animals of each group were housed together. Mice of positive and negative control groups were administrated with cyclophosphamide (40 mg/kg body weight) and physiological saline, respectively. The mice of melamine group were administered with melamine alone, at doses of 412, 824, and 1648 mg/kg/day corresponding the 1/8, 1/4 and 1/2 of $LD_{50\ oral}$ for melamine in male mice, respectively. The mice of Mix groups were administered both melamine and cyanuric acid with doses of each at 206, 412, or 824 mg/kg/day. These doses were selected based on the $LD_{50\ oral}$ of melamine in male mice (3296 mg/kg) (Melnick et al., 1984), and referred to "Procedures and Methods for Toxicological Assessment on Food Safety" compiled by Ministry of Health, PR China (Ministry of Health, 2003). All administrations were made via gastric gavages for 5 consecutive days. Thirty-fifth days after the first administration, body weight all survival mice were measured, and then the mice were sacrificed by cervical dislocation, and the right epididymises were removed immediately from every mouse.

2.4. Histopathologic observation

Testes were fixed in 10% neutral buffered formalin for 12 h or more, subsequently dehydrated and embedded in paraffin wax. For light microscope examination, sections were made and stained using hematoxylin and eosin. For electron microscope observation, the testes were dissected out and cut into small pieces of approximate 1 mm^3 with razor blades. The testis tissues were fixed in 2.5% glutaraldehyde, and stained using uranyl acetate and processed using standard dehydration in graded ethanol. And then, the specimens were embedded in epoxy resin. Ultrathin sections were cut with a diatome diamond knife, were stained with uranyl acetate and lead citrate. The ultrathin sections were examined and photographed with a JEM-1010 transmission electron microscope (JEOL, Tokyo, Japan).

2.5. TUNEL analysis of spermatogenic cell apoptosis

The testicular tissues were fixed in 4% formalin for 4 h or more, and embedded in paraffin according to routine procedures. Spermatogenic cell apoptosis was examined by TUNEL assay according to the manufacturer's instructions. In light microscope, the apoptotic positive cells would exhibit a brown nuclear stain. We quantified the number of stained cells for each group via counting the number of TUNEL stained nuclei per seminiferous tubular, and apoptotic index (AI) was defined as the number of apoptotic TUNEL-positive cells per 100 tubules.