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Acute testicular toxicity induced by melamine alone or a mixture of melamine and cyanuric acid in mice

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ABSTRACT

Eight-week-old male Kunming mice were administered either melamine (MA, 30, 140, or 700 mg/kg/day), a melamine and cyanuric acid mixture (MC, each at 15, 70, or 350 mg/kg/day), or vehicle (control) for 3 consecutive days. Testicular toxicity was evaluated on days 1 and 5 after the final exposure. The testicular and epididymal weights and serum testosterone level were significantly decreased in the highest MC group (350 mg/kg/day). Histopathologically, both MA and MC caused obvious lesions in the testis and epididymis, with significant increases in sperm abnormalities. By TEM, the blood–testis barrier was damaged dose dependently. TUNEL staining showed that both MA and MC induced increases in germ cell apoptosis. The Sertoli cell vimentin was collapsed in the treated animals as detected by immunohistochemical staining and Western blotting. This study demonstrated that both MA and MC treatments could disrupt the blood–testis barrier and cause a clear testicular toxicity.

1. Introduction

In September 2008, an outbreak of urinary stones in infants and children occurred in China because of consumption of melamine-contaminated powered formula. According to a report by The Ministry of Health of the People's Republic of China, a total of 294,000 infants and young children were affected, more than 50,000 of them were hospitalized, and six died from acute renal failure [1–3]. Early in 2004 and 2007, it had been reported that a large number of pets in the US, Canada, and South Africa died of acute kidney failure after ingestion of pet food contaminated with melamine and its analogs, especially cyanuric acid [4–7]. Melamine and its analogs are nitrogen-rich triazine compounds, and were added to food illegally to falsely boost the apparent protein levels. Because of the large potential health impact, a World Health Organization (WHO) expert meeting in collaboration with the Food and Agriculture Organization (FAO) was conducted to review the toxicity of melamine alone and in combination with cyanuric acid [8].

Early research on melamine toxicity showed that it had a low toxicity and mainly produced damage in the urinary organs [9–11]. Recently, a number of animal studies have clearly demonstrated that the toxicity of melamine will be dramatically boosted when combined with cyanuric acid, because the two compounds form an insoluble precipitate in kidney tubules that are of sufficient severity to cause renal failure via physical blockage [12–14].

However, the effects of melamine and its analogs on the other organs should not be neglected. Recommendations by WHO for further research included "Conduct studies to investigate potential reproductive and developmental effects" [8].

Several recent studies have reported on the potential testicular toxicity of exposure to melamine alone or in combination with cyanuric acid. One study [15] suggested that melamine had the ability to increase the sperm abnormality rate and DNA damage in mice. A few reports [16–18] showed that co-exposure to both melamine and cyanuric acid could cause testicular injury in mice. In our preliminary experiment, melamine crystals were found in the lumens of seminiferous tubules; this important finding made us speculate that melamine and its analog may have the ability to cross the blood–testis barrier from blood circulation. Therefore, studies are needed urgently to understand the testicular toxicity of melamine and its analog.

In the present study, we investigated the testicular toxicity of melamine alone and a mixture of melamine and cyanuric acid with multiple endpoints in mice.

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2. Materials and methods

2.1. Chemicals

Melamine (MA, purity > 99%, CAS 108-78-1) and cyanuric acid (CA, purity > 98%, CAS 108-80-5) were obtained from Beijing J & K Technology Co. Ltd. (Beijing, PR China). Carboxymethylcellulose (CMC) sodium was purchased from Sinopharm Chemical Reagent Beijing Co. Ltd. (Beijing, PR China). All other chemicals used in the study were of analytical grade or higher.

2.2. Animals and treatments

Adult male Kunming mice aged 7 weeks were purchased from the Laboratory Animal Center of Military Medical Sciences (Beijing, PR China). The animals were housed at 21–24 °C with a 12/12 h light/dark cycle for an acclimation period of 1 week before the experiment. Mice were given standard diet and tap water ad libitum. All experimental procedures were approved by the Institutional Animal Care and Committee of China Agricultural University.

Mice at 8 weeks of age were divided randomly into two experimental groups and a control group with 12 animals per group. The animals in the experimental groups were administered MA alone at doses of 30, 140, and 700 mg/kg/day or a mixture of MA and CA (MC) at 15, 70, and 350 mg/kg/day of each compound (for 15 mg/kg/day MC, =15 mg/kg/day MA + 15 mg/kg/day CA). The dosages were selected based on our preliminary study. These compounds were given as a suspension in 1% CMC once a day by oral gavage for 3 consecutive days. Control animals received 1% CMC in the same manner. Clinical signs and body weights were recorded daily.

Groups of animals were euthanized after the last dose at 1 and 5 days, respectively (each end point, $n = 6$). Blood samples were collected for biochemistry analysis. Epididymal sperm suspensions were collected for morphological observation. Organ samples were weighted and fixed in 2.5% paraformaldehyde–glutaraldehyde solution for morphological examination and immunohistochemistry analysis. One testis from each mouse was frozen in liquid nitrogen and stored at −80 °C for Western blotting (WB) analysis.

2.3. Blood biochemistry and testosterone measurement

The blood samples were allowed to clot and then were centrifuged. The serum was removed and frozen at −80 °C until clinical chemistry analysis by the Sino-uk Institute of Biological Technology, Beijing. Blood urea nitrogen (BUN) and creatinine (Cr) levels, serum markers of renal function, were assayed using an automatic biochemical analyzer.

Testosterone (T) levels in the serum were measured using radioimmunoassay (RIA) kits according to the manufacturer's protocols supplied by Sino-uk Institute of Biological Technology. This method is based on competition of serum testosterone with a testosterone–horseradish peroxidase conjugate for binding sites on an antibody-coated plate after extraction of testosterone by diethyl ether. The concentrations were calculated from a calibration curve using authentic testosterone. The intra-assay coefficient of variation was 7.4%, based upon the variability of the standard curve and the inter-assay coefficient of variation was 9.5%. Cross-reactivity with estriol, androstenedione, progesterone, and dihydrotestosterone was less than 1%. The limits of detection of the RIAs were 2 ng/dL testosterone.

2.4. Sperm abnormality determination

Spermatozoa were collected as quickly as possible after a mouse was sacrificed. The cauda epididymidis was cut to release spermatozoa into 1 mL of phosphate-buffered saline (pH 7.4) solution at 37 °C. The sperm solution was smeared onto glass slides and dried overnight. The slides were stained with 1% eosin Y. A total of 300 intact sperm were examined for each animal for morphological abnormality under the microscope. Abnormal heads were classified as straight, banana-shaped, or other unclassified abnormalities according to the method of Mori et al. [19].

2.5. Histopathology examination

Fixed testes and epididymides in 2.5% paraformaldehyde–glutaraldehyde were processed and embedded in paraffin wax, sectioned at 4 nm, and then stained with hematoxylin–eosin (HE) for light microscopy (Olympus, Japan). Testicular and cauda epididymal histology was evaluated according to the criteria of Oakberg [20] and Hess [21] with slight modifications.

The criteria for testicular score numbers and histopathological changes were: (0), minimal changes, <5% of tubules affected; (1), slight changes, 5–25% tubules affected; (2), moderate changes, 25–50% tubules affected; (3), marked changes, 50–75% tubules affected; (4), severe changes, >75% tubules affected. The criteria for epididymal score numbers and histopathological changes were: (0) no observable effect; (1) slight changes, normal sperm concentration 5–10 necrotic cells in the efferent ductules; (2) moderate changes, moderate decrease and 11–50 necrotic cells; (3) marked changes, marked decrease and >50 necrotic cells; (4) severe changes with a remarkable decrease in sperm concentration or azoospermia in the efferent ductules.

The diameters of seminiferous tubules were evaluated at 400× magnification using the Motic Med 6.0 CMIAS Image Analysis System (Motic China Group Co., Ltd., Beijing, PR China). For this purpose, 30 tubular profiles that were round or nearly round were chosen randomly and measured for each animal.

Table 1
Serum levels of BUN, CR and T at day 1 and day 5 in mice after dosed with MA alone or MC mixture for 3 consecutive days.

Groups	BUN (mmol/L)		CR (μmol/L)		T (ng/mL)	
	1 day	5 days	1 day	5 days	1 day	5 days
Con	7.17 ± 0.38[a]	7.61 ± 1.91	71.88 ± 0.97	58.29 ± 1.97	0.74 ± 0.16	0.47 ± 0.44
MA30	7.26 ± 0.89	7.32 ± 1.10	72.01 ± 3.31	57.34 ± 2.04	0.59 ± 0.15	0.61 ± 0.72
MA140	8.27 ± 0.50*	7.90 ± 0.99	69.39 ± 3.28	57.70 ± 0.89	0.65 ± 0.23	3.65 ± 2.09**
MA700	10.03 ± 5.11	7.90 ± 1.28	69.21 ± 1.31	58.76 ± 1.45	0.74 ± 0.11	0.65 ± 0.37
MC15	8.73 ± 0.84*	9.48 ± 1.19	70.36 ± 0.69	57.58 ± 2.81	0.80 ± 0.13	1.63 ± 1.86
MC70	16.89 ± 15.90**	11.22 ± 5.42	72.97 ± 1.09	59.54 ± 1.17	0.61 ± 0.11	0.49 ± 0.19
MC350	58.78 ± 13.28**	43.98 ± 27.82*	72.71 ± 1.43	64.51 ± 7.60	0.46 ± 0.12**	1.41 ± 2.08

Abbreviations: BUN, blood urea nitrogen; CR, creatinine; T, testosterone.

[a] Data are expressed as mean ± standard deviation ($n = 6$).

* Significant difference from the control group at $P < 0.05$.

** Significant difference from the control group at $P < 0.01$.