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Effect on morphology, oxidative stress and energy metabolism enzymes in the testes of mice after a 13-week oral administration of melamine and cyanuric acid combination

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ABSTRACT

Cases of pet poisoning and infant renal calculus have attracted much attention to the toxicity of melamine and its derivatives, such as cyanuric acid. Although individually melamine and cyanuric acid have low toxicity, their simultaneous presence can cause severe damage. Little is known about their adverse effects on the reproductive system. In this study, mice were orally administrated 1, 5 or 25 mg/kg/d of both melamine and cyanuric acid for 13 weeks. Lethargy, rough hair, and reduction of food and water intake and of body and testis weight were found after exposure to the combination, and pathological changes were found in the morphology of the testes, such as disruption of the seminiferous tubule structure, decrease of the spermatogenic cell series and coagulation necrosis. Total antioxidant capacity and superoxide dismutase activities and glutathione concentration was lower and malondialdehyde concentration was higher than in control mice. The activities of malate dehydrogenase, lactate dehydrogenase and Na^+/K^+-ATPase were also lower in combination treated mice than in control mice. These results indicate that the combined exposure to both melamine and cyanuric acid damaged testes in mice by either a direct or indirect effect, which may be related to renal failure and secondary anorexia. Oxidative stress and lower energy production levels both contributed to the testicular damage.

1. Introduction

Melamine is a nitrogen heterocyclic triazine compound that is widely used as an industrial chemical in the production of plastic, coatings, glue and flame retardants. Cyanuric acid is structurally related to melamine and is used as stabilizer. Both compounds have low toxicity (Mast et al., 1983; Melnick et al., 1984; Puschner et al., 2007). However, when both are present the combination is more toxic (Gamboa da Costa et al., 2012; Jacob et al., 2011; Park et al., 2011; Puschner et al., 2007). The two compounds may be combined intentionally because their high nitrogen content elevates the apparent protein content of food, or they can be combined unintentionally because cyanuric acid is a byproduct of melamine synthesis (Guan et al., 2009). Until now, research on the toxicity of the combination of melamine and cyanuric acid has mainly focused on lesions to the kidney, including formation of crystals in renal tubules, necrosis in the tubular epithelia, renal interstitial edema, and increases in blood urea nitrogen and creatinine (Kim et al., 2011; Ogasawara et al., 1995; Okumura et al., 1992; Park et al., 2011; Puschner et al., 2007). A recent study has shown that melamine can induce sperm abnormality and sperm DNA damage (Zhang et al., 2011), suggesting that the coexistence of melamine and cyanuric acid may cause damage to the male reproductive system.

Oxidative stress results from an imbalance between reactive oxygen species (ROS) production and the anti-oxidative activity of enzymes such as catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase and numerous non-enzymatic antioxidants (Schafer and Buettner, 2001). It has been reported that oxidative stress has a critical role in testis damage (Aydilek et al., 2004; Khan et al., 2011). Excessive ROS can damage lipids, fatty acids, proteins (protein carbonyl formation) and DNA, leading to structural and functional disruption of the cell membrane, inactivation of enzymes and cell death (Cooke et al., 2006; Gałazyn-Sidorczuk et al., 2009; Moshahid Khan et al., 2012). The testis executes high-energy-demanding processes such as spermatogenesis and steroidogenesis, and the activities of energy metabolism enzymes, such as ATPase, lactate dehydrogenase (LDH) and sorbitol dehydrogenase (SDH), are important for testicular cells (Abdul-Ghani et al., 2008; Yan et al., 2010). Disturbances to energy metabolism may cause damage to testis cells. There have been no reports so far about the combined toxicity of melamine and cyanuric acid to the reproductive system. The aim of the present study is to analyze the effect of combined exposure to melamine and cyanuric acid for

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13 weeks on the morphology, oxidative stress and energy metabolism enzymes of mouse testes.

2. Materials and methods

2.1. Animals and treatment

ICR male mice (21 days old) were purchased from the Laboratory Animal Center of Nanjing Medical University (Nanjing, China). Mice were housed in separate cages at a temperature of 21 ± 1 °C and given a standard mice chow and water *ad libitum*. There were no melamine and cyanuric acid in the chow and water detected by gas chromatography/tandem mass spectrometry with reference to the previous studies (Miao et al., 2009; Qin et al., 2010). After 1 week acclimation to the laboratory environment, the mice were randomly assigned to four groups of 10 mice each and were each orally given 0, 1, 5, or 25 mg/kg/d of melamine plus the same amount of cyanuric acid dissolved in corn oil for 13 weeks. The mice were weighed and euthanized after the last treatment. The testes were collected and weighed and the relative weight of the testes was calculated as a proportion of each animal's body weight. Five mice testes were fixed in 10% formaldehyde, and the other five mice testes were stored at −80 °C. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University.

2.2. Histopathological analysis

Samples of the testes which fixed in 10% formaldehyde were dehydrated by using a series of alcohols, clarified in xylene, and embedded in paraffin. Then samples were sliced serially into 4 μm sections and stained with hematoxylin and eosin using routine methods. Stained sections were examined with a light microscope (Zeiss, Germany).

2.3. Tissue preparation

The testes tissues were weighted, homogenized in 0.9% NaCl to a final concentration of 10% homogenate, and then centrifuged at 3000 rpm at 4 °C for 15 min. The supernatants were collected and the concentration of total protein was estimated by the bicinchoninic acid (BCA) method with bovine serum albumin as a standard using a Pierce BCA protein assay kit (Thermo scientific, USA).

2.4. Antioxidant enzyme determination

The total antioxidant capacity (TAC) was measured using a Total Antioxidant Assay Kit from the Beyotime Institute of Biotechnology (Haimen, China). The TAC activity was measured based on the ability of antioxidants to inhibit the oxidation of ASTS [2, 2′-azinodi-(3-ethylbenzthiazoline sulphonate)] to $ABST^{+}$ by metmyoglobin. Briefly, a 10 μl sample of supernatant from the testes was placed in a 96-well plate. Serial dilutions of Trolox (6-hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid) standards were also used. Then, a 20 μl sample of myoglobin working solution was added to each well with a sample and 150 μl ASTS working solution was placed into each well. After 5 min of incubation at room temperature, the formation of a blue–green color was measured at an optical density of 600 nm. The results were expressed as the Trolox concentration (nmol) per milligram of total protein. SOD activity and GSH concentration were measured using kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The SOD activity was measured based on the extent inhibition of amino blue tetrazolium formation in the mixture of nicotineamide adenine dinucleotide, phenazine methosulphate and nitroblue tetrazolium (NADH–PMS–NBT). Briefly, 10 μl sample of testes supernatant was added to 130 μl mixture that contained 300 μM NBT. After the mixture was incubated at 37 °C for 90 s, the reaction was stopped by the addition of 1 ml of glacial acid. The reaction mixture was stirred vigorously with n-butanol and the color intensity of the chromogen in the butanol phase was measured spectrophotometrically at 550 nm. One unit of enzyme activity was defined as the amount of enzyme that caused a 50% inhibition of the NBT reduction per milligram of protein. The concentration of GSH was based on the development of a yellow color when DTNB (5,5-dithiobtis-2 nitrobenzoic acid) was added to compounds containing sulfhydryl groups. A 500 μl sample of homogenate from the testes was added to 3 ml of 4% sulfosalicylic acid. The mixture was centrifuged at 1000 rpm for 10 min and 100 μl supernatant was then taken and added to the DNTB solution. The absorbance was measured at 405 nm after 5 min. The concentration of GSH was calculated using GSH (Sigma) as a standard and GSH content was expressed as μmol/mg protein.

2.5. Malondialdehyde and protein thiol group determination

The concentrations of malondialdehyde (MDA) and protein thiol groups (PSH) were measured using kits from Nanjing Jiancheng Bioengineering Institute. MDA reacts with thiobarbituric acid (TBA) to form a red product, which was measured by a spectrophotometer. Briefly, 0.1 ml of 0.67% TBA was added to 0.1 ml testes supernatant. This was then heated in a tightly stoppered tube for 40 min in a boiling water bath. After cooling, the tubes were centrifuged at 3500 rpm for 10 min to separate the precipitated proteins. The absorbance of any red color produced was read at 532 nm. The amount of MDA produced was calculated using 1,3,3-tetraethoxy propane as a standard and the result of peroxidation was expressed as the nanomolar concentration of MDA formed per mg of protein. The concentration of PSH was measured based on their reaction with DTNB (5, 5-dithiobis-2-nitrobenzoic acid) to form a yellow dye. Briefly, 250 μl testes supernatant was mixed with 750 μl of 0.2 M trichloroacetic acid. After centrifugation, the protein pellet was discarded and free-SH groups were determined in a clear supernatant. A 10-μl aliquot of supernatant was added to 85 μl of 1 M potassium phosphate buffer (pH 7.4) and 5 μl of 10 mM DTNB. The absorbance of the yellow color produced was read at 405 nm. PSH concentration was calculated using a standard curve generated with GSH and the results were calculated as μmol/g protein.

2.6. Malate dehydrogenase, LDH and Na^{+}/K^{+}-ATPase determination

The activities of malate dehydrogenase (MDH) and LDH were measured with kits from Nanjing Jiancheng Bioengineering Institute. The activity of MDH was measured spectrophotometrically by following the oxidation of reduced pyridine nucleotides with oxaloacetate. Briefly, 1 ml reaction mixture which contained sodium oxaloacetate and NADHP was incubated for 3 min at 37 °C, and 50 μl testes supernatant was then added to the mixture. Reduced pyridine nucleotide oxidation was measured at 340 nm at 20 and 80 s. One unit of enzyme activity was defined as the volume of 1 μmol reduced pyridine nucleotides oxidized in 1 min. The results were expressed as U/mg protein. The LDH activity was based on the principle that LDH catalyzes the oxidation of NAD to NADH. NADH and pyruvate were added and the testicular supernatant was incubated at 37 °C for 15 min. Next, the samples were incubated with the coloring reagent at 37 °C for 15 min. The reaction was stopped by the addition of 0.4 mol/L NaOH. LDH activity was proportional to the increase in absorbance due to the reduction of NAD to NADH. The absorbance was determined at 450 nm.