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Melamine negatively affects testosterone synthesis in mice

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ABSTRACT

Several studies have found that melamine causes damage to the testes, epididymis and sperm. However, few studies have investigated the effect of melamine on the synthesis of testosterone, which plays an import role in testicular development and spermatogenesis. In present study, mice were orally administrated with 2, 10 or 50 mg/kg of melamine for 28 days. In these groups, various abnormalities were observed including disruption of the seminiferous tubule structure, an increased necrotic germ cells and sperm abnormalities, and a reduced sperm count. Melamine exposure also decreased the level of serum testosterone and levels of testicular StAR, P450scc and 17β-HSD. In addition, melamine exposure reduced the number of Leydig cells. Taken together, these results indicate that melamine exposure reduces the level of testosterone through down-regulation of StAR and testosterone synthetic enzyme expression and also a decreased number of Leydig cells. This may further affect testicular development and lead to sperm damage.

1. Introduction

Melamine is widely used in industry in the manufacture of plastic, fabrics, laminates, food contact materials and tableware products. Because of its high nitrogen content, melamine has been added illegally to human and animal food. Two high-profile examples are the 2007 pet food recall due to melamine contamination in the USA and the 2008 infant renal calculus cases in China (Brown et al., 2007; Guan et al., 2009), both of which led to our interest in melamine. In addition, due to its use in food contact products, melamine can leach into food, a process which is accelerated by high temperatures or when acetic acid is present (Chik et al., 2011; Lynch et al., 2015). It has been reported that consumption of foods contained in melamine bowls increased urinary melamine levels (Wu et al., 2013) and therefore the potential risk of melamine also should be considered.

Previous studies of melamine toxicity have focused mainly on the urinary system, including the increase in concentration of urea and creatine, crystal formation, inflammatory cell infiltration and kidney failure (Kobayashi et al., 2010; Chen et al., 2014). Recently, our and other studies have demonstrated that melamine can cause lesions in the male reproductive organs, including tissue disorganization, necrosis and apoptosis of spermatogenic cells, disruption of the blood-testis barrier and necrosis of germ cells in the epididymis (Lv et al., 2013; Yin et al., 2013; Chang et al., 2014). Melamine has also been found to damage sperm, resulting in a reduced sperm count, an increased number of abnormalities and DNA damage (Zhang et al., 2011; Yin et al., 2013; Chang et al., 2014).

Since testosterone (T) is essential for normal testicular development and spermatogenesis, it is possible that these effects are due to the effect of melamine on testosterone production. Since there are currently few studies on the effect of melamine on testosterone synthesis, the present study investigates the change in testosterone level in mice after exposure to melamine. The production of testosterone in the Leydig cells is influenced by the steroidogenic acute regulatory (StAR) protein and testosterone synthetic enzymes including cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc), cytochrome P450 17α-hydroxysteroid dehydrogenase (P45017α) and 17β-hydroxysteroid dehydrogenase (17β-HSD); thus the number of Leydig cells and the change of StAR and testosterone synthetic enzymes were also studied.

2. Materials and methods

2.1. Animals and treatment

Forty ICR male mice aged 4 weeks were purchased from the Laboratory Animal Center of Nanjing Medical University (Nanjing, 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 a standard diet and ad lib tap water. At 4 weeks of age, mice were divided randomly into four groups of 10 mice per group. Three groups were given melamine (MA, Shanghai ANPEL Laboratory Technologies Inc., Shanghai, PR China) at doses of 2, 10 or 50 mg/kg/day (MA2, MA10, MA50, respectively), as previous studies (Lv et al., 2013; Yin et al., 2013). These compounds were given as a suspension in edible oil once a day by oral gavage for 28 consecutive days. Control mice received

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edible oil only in the same manner. Clinical signs and body weights were recorded daily after administration with MA or edible oil. After the final day of treatment, blood samples were collected from the retro-orbital sinus before mice were euthanized by CO_2 inhalation in designated CO_2 chambers. The gross changes of kidneys were examined. The testes were collected and weighed, and the relative weights of the testes were calculated as a proportion of each animal's body weight. Ten right testes were fixed in 10% formaldehyde for histopathological examination and immunohistochemical analysis. Ten left testes were stored at −80 °C for real-time PCR and Western blotting analysis. Epididymides were also collected for sperm morphological observation and abnormality counting. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Nanjing Agricultural University.

2.2. Sperm number and abnormality determination

Spermatozoa were collected as quickly as possible after the mice were euthanized. Briefly, the cauda epididymis was cut to release spermatozoa into 5 mL of phosphate-buffered saline (pH 7.4) solution at 37 °C. The number of sperm in each mouse was counted under light microscope (Zeiss, Germany). The sperm solution was smeared onto glass slides and fixed with methanol. After being air-dried overnight, the smears were stained with 2% aqueous eosin solution for 1 h and washed gently with distilled water. A total of 1000 intact sperm from each mouse were examined for morphological abnormality under the light microscope. Spermatozoa with the following deformities were counted as abnormal: banana-shaped head, no head, double head, neck torsion, amorphous head, no hook, fold in middle and tail, fat head, and coiled tail (Mori et al., 1991; Yin et al., 2013). This abnormality rate was calculated as a proportion of 1000 sperm.

2.3. Histopathological examination

The testes were fixed in 10% formaldehyde and dehydrated by 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 by routine methods. Stained sections were examined with a light microscope.

2.4. Serum testosterone detection

Blood samples were collected and centrifuged at 3000 rpm to obtain serum. Testosterone levels in the serum were measured using radioimmunoassay (RIA) kits supplied by Beijing BeiFang Biological Technology Co. Ltd. (Beijing, China) according to the manufacturer's protocols.

2.5. Immunohistochemical examination

The testicular paraffin sections were deparaffinized and dehydrated, and antigens were exposed by microwave heating in 10 mM sodium citrate solution for 10 min. Sections were pretreated with 3% H_2O_2 and 10% normal bovine serum for 30 min each, and then they were incubated overnight in the primary 3β-HSD antibody (Santa Cruz Biotechnology, Dallas, Texas, USA) at 4 °C. Then slides were incubated with horseradish peroxidase-conjugated horse anti-goat IgG antibody for 1 h at 37 °C. The sections were then treated with DAB for 3-5 min, counterstained with hematoxylin, and mounted with gum. The number of 3β-HSD-positive cells was counted in 12 randomly selected fields from each slide at a magnification of 400× according to previous studies (Ji et al., 2010; Zhang et al., 2009).

2.6. Quantitative real-time PCR detection

The mRNA levels of StAR, P450scc, P45017α and 17β-HSD were measured according to our previous study (Sun et al., 2016). β-actin was used for the housekeeping gene. The PCR primers were designed as previously reported in Genbank, and the primer sequences for these genes were:

StAR Sense: 5′ AAGGAAAGCCAGCAGGAGAAC 3′

Anti-sense: 5′ TCCATGCGGTCCACAAGTT 3′
Excepted PCR product size: 135 bp
P450scc Sense: 5′<!–[/INS] CCATCAGATGCAGAGTTTCCAA 3′
Anti-sense: 5′TGAGAAGAGTATCGACGCATCCT 3′
Excepted PCR product size: 110 bp
P45017α Sense CCATCCCGAAGGACACACAT
Anti-sense:CTGGCTGGTCCCATTCATTT
Excepted PCR product size: 100 bp
17β-HSD Sense:ATGCCCTCCTGGCTCCTT
Anti-sense:CACCCACAGCGTTCAATT
Excepted PCR product size: 167 bp
β-Actin Sense:GAGACCTTCAACACCCCAGC
Anti-sense:ATGTCACGCACGATTTCCC
Excepted PCR product size: 236 bp

2.7. Western blotting

The left testes from each animal were homogenized in RIPA buffer (Beyotime, Shanghai, China). After centrifugation at 14,000 g for 5 min at 4 °C, the supernatants were collected and the protein concentrations were determined using the BCA protein assay kit (Thermo, Rockford, IL, USA). Equal amounts of sample proteins (20 μg) were loaded on a 10% sodium dodecylsulfate-polyacrylamide gel. Samples were transferred to polyvinylidene fluoride membranes, which were blocked for 1 h with 5% nonfat dry skim milk suspended in 0.1% Tween-20 Tris-buffered saline (TBST, pH 7.4). Membranes were then incubated overnight at 4 °C with an anti-StAR primary antibody (Santa Cruz Biotechnology, Dallas, Texas, USA), P450scc (Santa Cruz), P45017α (Santa Cruz), 17β-HSD (Santa Cruz), glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Abcam, Shanghai, China) or β-tubulin (Enogene,Nanjing, China). Membranes were washed in TBST, and then incubated with the second antibody (SunshineBio, Nanjing, China), horseradish peroxidase-conjugated secondary antibody at room temperature for 2 h and developed using enhanced chemiluminescence regents. Signals were captured and measured using the Image Quant LAS 4000 biomolecular imager.

2.8. Statistical analysis

Results are expressed as the mean ± standard deviation (SD). Differences between groups were determined with a one-way analysis of variance (ANOVA) followed by the LSD multiple comparison test, using SPSS version 20.0 for Windows. *P*-values of 0.05 or 0.01 were taken to be statistically significant, and are labeled with an asterisk (* or **, respectively) on each graph.

3. Results

3.1. Clinical observation and weight change of body and testes

The mice in the control, MA2 and MA10 groups grew steadily and no clinical changes were observed. However, the mice in the MA50 group showed a mild clinical change including depression and hunched back. There was no significant difference in food and water intake (data not shown) or body weight (Fig. 1A) between the MA-treated and control groups ($P > 0.05$). No mice died during the MA administration period. After euthanasia, no obvious gross lesions were found in the kidneys or testes, and there was no significant difference in testis weight ($P > 0.05$, Fig. 1B).