Effect of Melamine on Immunohistochemical Expression of Bax/Bc1-2 Protein in Testis and ER-α/PR mRNA in Ovary With or Without Cyanuric Acid in Mice

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ABSTRACT

Melamine is a nitrogen heterocyclic triazine compound whose acute toxicity was considered to be low in mammals. The combined ingestion of melamine and cyanuric acid was shown be responsible for the crystalluria, kidney stones and subsequent renal failure in animals. In the present study, we investigated in mice the potential effects of melamine on immunohistochemical expression of Bax/Bc1-2 protein in testis and the estrogen receptor-α (ER)/progesterone receptor (PR) mRNA in the ovary with or without cyanuric acid. Our results indicated that at the dose range used, exposure to melamine alone or combination with cyanuric acid promoted the expression of Bax protein, and suppressed the expression of Bcl-2 protein in testis of male mice in a dose-dependent manner. The relative expression of *ER*-α in ovary was significantly down-regulated in the animals from the melamine high dose group (50 mg/kg), as well as in the mixture groups of melamine and cyanuric acid at low (each at 1 mg/kg), middle (each at 5 mg/kg) and high doses (each at 25 mg/kg). The changing pattern of PR mRNA in the animals treated with melamine alone or combination of melamine and cyanuric acid were similar to those of ER-α mRNA. Compared with control group, however, no significant difference was observed in relative expression of PR mRNA in co-administration group of melamine and cyanuric acid with low dose (each at 1 mg/kg, $P > 0.05$). These results from the present study may provide useful information for evaluating the melamine-related reproductive toxicity in mammals.

Keywords: Mouse, Melamine, Cyanuric acid, Bax/Bc1-2, Testis, ER-α/PR, Ovary.

INTRODUCTION

Melamine, a nitrogen heterocyclic triazine compound, is extensively used in the production of plastics, glues, kitchenware, commercial filters, dishware and fabrics (1, 2). When added to the foodstuffs it falsely elevates the apparent protein concentration due to its high nitrogen content (approximately 66 % by molecular weight) (3). Thus, melamine was found to be illicitly mixed into pet food and milk to increase the apparent protein concentration (4, 5). Although, the acute toxicity of melamine alone was shown to be low in mammals (6, 7), the combination of melamine with cyanuric acid was considered to be responsible for the crystalluria, kidney stones and subsequent renal failure in animals (8). Therefore, the renal toxicity of melamine has become an area of concern to nephrologists (8).

In recent years, however, it has been increasingly appreciated that the toxicity of melamine might not be limited to the kidneys (8). It was demonstrated that melamine not only inhibits the proliferation of differentiated PC12 cells through the induction of apoptosis (9), but also affects the

morphology and caspase-3 activity of hippocampus neurons (10). Also, it was reported that melamine may cause sperm deformity and DNA damage (11, 14). Additionally, Xie *et al.,* (2011) demonstrated that the co-administration of melamine and cyanuric acid resulted in damage to the liver in mice in a dose-dependent pattern (13). In our recent study, we also demonstrated that melamine resulted in certain ultrastructural pathological injuries to the liver, kidney, spleen, stomach wall, and small intestine of mice (12).

The reproductive system is more sensitive to toxic chemicals in comparison to other system (15, 16). Testis and ovary are the primary organs of the reproductive system in mammals. It was reported that melamine alone or its combination with cyanuric acid resulted in the damage to the testis of mice (14, 17), and the melamine-related toxicity was found to be related to germ cell apoptosis (18). However, it is unclear whether the increase in apoptotic index of the germ cells is a direct or indirect effect (12, 17).

Bax and *Bcl-2* are two crucial genes participating in apoptotic process. Bax protein promotes the apoptotic cell death through inserting into the mitochondrial membrane and increasing membrane permeability (19, 20), whereas Bcl-2 protein prevents this process by preserving mitochondrial integrity (21). Therefore, the balance between Bax and Bcl-2 is crucial for the induction of apoptosis.

There is a dearth of information about the toxic effects of melamine alone or its combination with cyanuric acid on the ovary in mammals. Through investigation of gene knockout mice it has been demonstrated that estrogen receptor-α (ER-α) and progesterone receptor (PR) play critical roles in the regulation of the reproductive physiology of female mice (22, 23).

The purpose of the present study was to investigate the potential effects of melamine on immunohistochemical expression of Bax/Bc1-2 protein in testis and ER-α/PR mRNA in ovary with and without cyanuric acid in mice.

MATERIALS AND METHODS

Animals

A total of 56 healthy male mice of the Kunming strain (3 week-old, 25-30 g) were used provided by Beijing Fukang Biological Technology Co., Ltd. (license No.: SCXK (Jing) 2009-0004, Beijing, China). Another 56 healthy female mice of the Kunming strain (3 week-old, 25-30 g) were obtained from China Medical University (license No.: SCXK (Liao) 2008-0005, Shenyang, China), All animal protocols were reviewed and approved by the Animal Experimental Committee of Shenyang Agricultural University.

Animal grouping and material administration

The mice were maintained in controlled laboratory conditions of 12 h dark/light cycle and 22±2˚C temperature with a relative humidity of 35-65%. After seven days of acclimation, the male mice were randomly divided into seven groups of eight mice each, i.e. one control group, three melamine groups (low, middle, and high doses) and three mixture group of melamine and cyanuric acid (low, middle, and high doses). The eight animals of each group were housed together. The animals had free access to water and standard laboratory food (containing 24% protein, 4% fat and about 5% fiber) that was provided by the experimental animal center of Liaoning University of Traditional Chinese Medicine (Shenyang, China). All feeds and water were subjected for the detection of melamine or cyanuric acid contaminants as the described by Heller *et al.* (2008) (24). Neither melamine nor cyanuric acid was detected above the limit of 0.5 ppm.

The mice of melamine group were administered with melamine (> 99%, Sinopharm Chemical Reagent Beijing Co., Ltd, Beijing, China) at doses of 2 mg/kg (low dose), 10 mg/kg (middle dose) and 50 mg/kg (high dose) twice daily. The mice of mixture groups were administered with the combination of melamine and cyanuric acid (> 98%, Shanghai Crystal Pure Industrial Co., Ltd, Shanghai, China) at the dosages consisting of 1 mg/kg (low dose), 5 mg/kg (middle dose) and 25 mg/kg (high dose) twice daily. The mice of control group were administered 1 mL of physiological saline. All administrations were performed via gastric gavages for 30 days. At the end of the experiment, all mice of each group were sacrificed by cervical dislocation, and the testis tissues of male mice and ovary tissue of female mice were collected immediately from each mouse.

Immunohistochemical detection of Bax and Bcl-2 protein in the testis of male mice

The testis tissue collected was fixed in 10% neutral formalin for 24 hours and then embedded in paraffin. Serial sections (4 μm) were cut and mounted on poly-l-lysine coated slides. The sections were deparaffinized and rehydrated routinely. The sections were incubated with 0.3% H_2O_2 for 30 min to

block endogenous peroxidase activity, and then were washed with distilled water for 3 times. Antigens were retrieved through heating the sections in a microwave oven (pH 6.0 for 20 min). Slides were allowed to cool, and subsequently were washed twice in PBS-buffer (pH 7.3). Slides were incubated with primary antibodies against Bax (Boster, Wuhan, China) or Bcl-2 (Boster, Wuhan, China) at 37 °C for 1 h. The slides were covered with coverslips in order to ensure that the whole section was coated with the antibody solution. After washing three times for 2 minutes with PBS (pH 7.3), the slides were further incubated with the secondary antibody. The reaction results were visualized using DAB chromogen. The results were analyzed using the JD-801 morphological microscopic image analysis system (JieDa Biotechnology Co. Ltd., Jiangsu, China).

Detection of ER-α/PR mRNA in ovary of female mice

Using Trizol reagent (Sangon, Shanghai, China), total RNA was extracted from the ovary tissue of the female mice following the manufacturer's instructions. The integrity of total RNA extracted was verified with 1.5% agarose gel electrophoresis. The purity and quantity of the extracted total RNA were assessed in an ultraviolet spectrometer with the ratio of OD_{260}/OD_{280} being 1.8 to 2.0 for each sample. The total RNA was treated with DNase to exclude the contamination from residual genomic DNA. Using M-MuLV cDNA Synthesis Kit (Sangon, Shanghai, China), the first strand cDNA was synthesized with 1μg of total RNA for each sample according to the manufacturer's instructions. A negative control was included for each sample without template RNA. The cDNA was diluted 1:20 with DNase/RNase free water. Four primers were designed according to the sequences of ER-α (NM_007956.4) and PR (NM_008829.2) mRNA in GenBank. Also, another pair of primers targeting the β-*actin* gene (accession number in GenBank: NM_007393.3) was designed as an internal control. The information on these primers is shown in Table 1. The specificity of the primer sets was confirmed by testing them on the first-stand cDNA obtained from this work in a preliminary PCR experiments with subsequent sequencing analysis.

We prepared the 10-fold dilution of cDNA obtained from ovary tissue total RNA, and produced a 6-point standard curve for each gene under analysis. The real-time PCR reaction was carried out in a 20 μL final volume containing 10 μL 2 × SYBR® Premix Ex Taq™ (TaKaRa, Dalian, China), 0.4 μL forward primer (10 μ*M*), 0.4 μL reverse primer (10 μ*M*), 2.0 μL first-strand cDNA, and 7.2 μL PCR grade water. Using SYBR Green I assay, the amplification was carried out in a LightCycler 480 Thermal Cycler (Roche Diagnostics, Germany) with the following program: 95°C for 4 min, then 40 cycles of the following: 95°C for 15s, 54-56 °C (Table 1) for 1 s, and 72°C for 20s. At the end of each real-time PCR, a melting curve analysis ranging from 65 to 95°C was performed for each sample to confirm the presence of one gene-specific single peak for each primer set. All reactions were run in triplicate. A negative control without cDNA template was included in each measurement. The real time PCR efficiency (*E*) were calculated by the given slopes from the instrument's software using the equation $E = 10^{(-1/\text{slope})}$.

Statistical analysis

Data was tested for normal distribution. Subsequently, using single factor analysis of variance (ANOVA), the statistical analysis was carried out by SAS 6.12 software (SAS Institute, Cary, NC, USA), and t-test was used as a *post hoc* test after the ANOVA. Data was presented as the mean ± standard error. $P < 0.05$ was considered as statistically significant.

Table 1: Information of primers used for analyzing the expression ER-α/PR mRNA in female ovary

Primer name	Gene[1]	Primer orientation	Sequence (5'–3')	Primer length (bp)	Annealing temperature (°C)	Amplicon size (bp)
mER-FW	ER-α	Sense	GCACCAGATCCAAGGGAA	18	56	150
mER-RV	ER-α	Anti-sense	GCGGCGTTGAACTCGTAG	18		
mPR-FW	PR	Sense	CTCATAGGGAAGGAGGCAGAA	21	55	155
mPR-RV	PR	Anti-sense	CCCAAAGAGACACCAGGAAGT	21		
mACTB-FW	β-actin	Sense	CTGTCCCTGTATGCCTCTG	19	54	221
mACTB-RV	β-actin	Anti-sense	TTGATGTCACGCACGATT	18		

[1] ER-α = Estrogen receptor- α, and PR = Progesterone receptor

RESULTS

Clinical observations

Throughout the experiment, no death was recorded for the male mice used for investigating immunohistochemical expression of Bax/Bc1-2 protein in testis. Only three female mice used for analyzing ER-α/PR mRNA in ovary, all of which received a co-administration of the high group of melamine and cyanuric acid (each at 25 mg/kg/day), died at days 19, 25 and 28, respectively, and they exhibited anorexia, dull hair coat, decreased activity and a hunched posture before death.

At the end of the experiment, the mice treated with the combination of melamine and cyanuric acid (25mg/kg/2 day) had a lower body weight versus the control group, but without statistical significance ($P > 0.05$). At necropsy, the kidneys of survival mice appeared pale yellow in color and were grossly enlarged. No changes were noted in the other organs including testis of male mice and ovaries of female mice.

Immunohistochemical changes of Bax and Bcl-2 protein in the testis in male mice

As observed from Figure 1, the positive expression of Bax protein was primarily observed in cytoplasm and cell membranes showing diffuse or linear staining. The animals from melamine-treated group with high dose (50 mg/kg/ 2 days) exhibited a strong positive reaction (Figure 1 D). Similar results were also observed in the animals from mixture groups of melamine and cyanuric acid with middle (each at 5 mg/kg) and high (each at 25 mg/kg) doses (Figure 1 F and G). However, other groups exhibited moderate or weak positive reactions (Figure 1 A, B, C and E).

On the other hand, the Bax protein was expressed mainly in the spermatogonia and primary spermatocytes of seminiferous tubules. Also, Bax protein was observed in spermatogenic cells at all levels for the mice from melamine-treated group with high dose (50 mg/kg/ 2 days), as well as mixture groups of melamine and cyanuric acid with middle (each at 5 mg/kg) and high (each at 25 mg/kg) doses. Additionally, Bax protein was found to be expressed abundantly even in the stromal cells of these mice.

As shown in Table 2, on the whole, the expression level of Bax protein was low in seminiferous tubules of the mice from control group. Compared with control group, however,

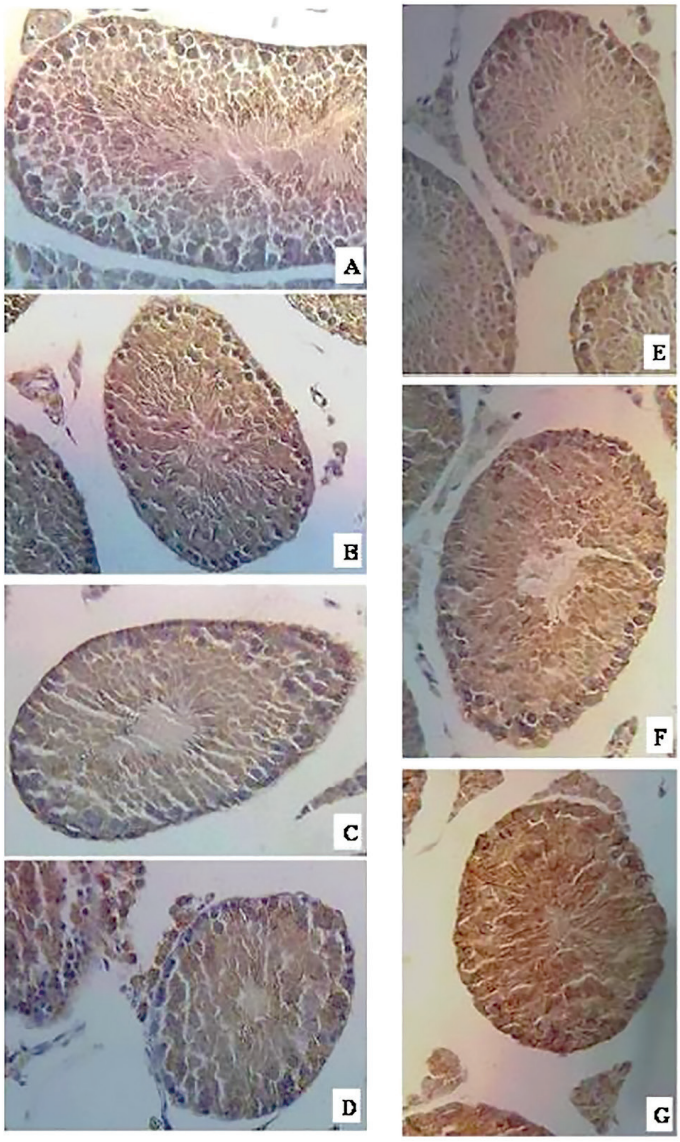

Figure 1: Testicular section from different treated groups showed expression changes of Bax protein in male mice that were detected by immunohistochemical assay (×200). (A) control group; (B) Melamine-treated group with low dose (2 mg/kg); (C) Melamine-treated groups with middle dose (10 mg/kg); (D) melamine-treated groups with high dose (50 mg/kg); (E) Co-adminstration of melamine and cyanuric acid with low dose (each at 1 mg/kg); (F) Co-adminstration of melamine and cyanuric acid with middle dosage (each at 5 mg/kg); (G) Co-adminstration of melamine and cyanuric acid with high dose (each at 25 mg/kg).

the average optical density value of Bax protein expression was significantly higher in the melamine-treated high dose group (50 mg/kg) compared to the control group ($P < 0.05$). Similarly, in comparison to the control group, the average optical density value of Bax protein expression had a significant increase in mixture group of melamine and cyanuric

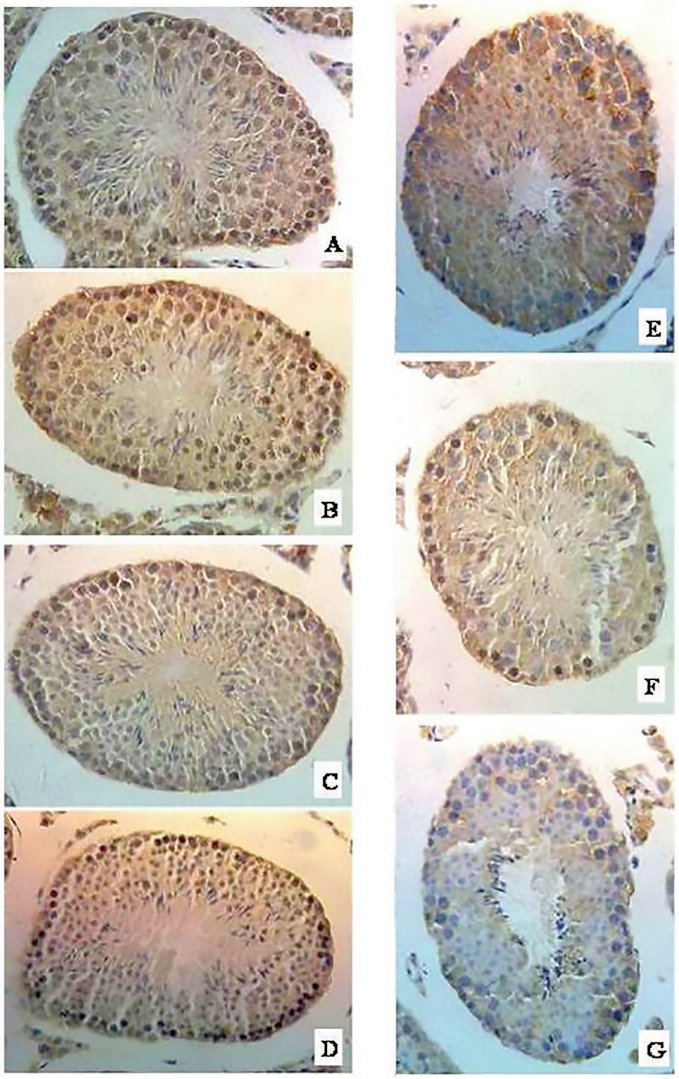

Figure 2: Testicular section from different treated groups showed expression changes of Bcl-2 protein in male mice that were detected by immunohistochemical assay (×200). (A) Control group; (B) Melamine low dose group (2 mg/kg); (C) Melamine middle dose groups (10 mg/kg); (D) Melamine high dose group (50 mg/kg); (E) Mix low dose group of melamine and cyanuric acid (each at 1 mg/kg); (F) Mix middle dose group of melamine and cyanuric acid (each at 5 mg/kg); (G) Mix high dose group of melamine and cyanuric acid (each at 25 mg/kg).

acid with both middle dose (each at 5 mg/kg) and high dose (each at 25 mg/kg/2 day, $P < 0.01$). Also, positive expression area of Bax protein in different degrees was observed with different doses of both melamine alone and combination of melamine and cyanuric acid in a dose-dependent manner. These results suggested that the ingestion of melamine alone or combination with cyanuric acid can promote the expression of Bax protein in testis of male mice.

Table 2: Effect of melamine on immunohistochemical expression of Bax/Bc1-2 protein in testis with or without cyanuric acid in mice

Groups	Optical density of Bax protein	Optical density Bc1-2 protein	Bax/Bc1-2
Control	0.3458±0.0532	0.3512±0.0468	0.98
Melamine (low, 2 mg/kg)	0.2869±0.0765	0.2882±0.0631	1.0
Melamine (moderate, 10 mg/kg)	0.3387±0.0496	0.2788±0.0584	1.21
Melamine (high,50 mg/kg)	0.3751±0.0753*	0.192±0.0759**	1.95
Mixture of melamine and cyanuric acid (low, 1 mg/kg/ 2 days)	0.3657±0.0895	0.3176±0.0438	1.15
Mixture of melamine and cyanuric acid (middle, 5 mg/kg/ 2 days)	0.4107±0.0326**	0.2432±0.0568*	1.69
Mixture of melamine and cyanuric acid (high, 25 mg/kg/ 2 days)	0.4575±0.0653**	0.1835±0.0732**	2.49

In comparison to control group, * indicates statistical significance difference at $P < 0.05$, and ** indicates statistical significance difference at $P < 0.01$.

As shown in Figure 2, the positive expression of Bcl-2 protein was observed in spermatogenic cells at all levels. The Bcl-2 protein was expressed mainly in the spermatogonia and primary spermatocytes of seminiferous tubules and stromal cells. In the animals from melamine-treated group, Bcl-2 protein was localized in the cytoplasm and cell membrane exhibiting a moderate or weak positive reaction. As shown in table 2, in comparison to control group, the average optical density value of Bcl-2 protein expression was significantly lower in the animals from melamine-treated group with high dose (50 mg/kg) compared to the control group ($P < 0.01$). Also, the co-administration of melamine and cyanuric resulted in a significantly lower average optical density value of Bcl-2 expression in the animals from both middle dose (each at 5 mg/kg) and high dose (each at 25 mg/kg, $P < 0.01$) compared with control group. A dose-dependent manner was observed in the average optical density value of Bcl-2 expression in the exposure to melamine alone or the combination with cyanuric acid. These results suggested that the ingestion of melamine alone or combination with cyanuric acid can suppress the expression of Bcl-2 protein in testis of male mice.

Additionally, in comparison to control group, a greater ratio of Bax against Bcl-2 was observed in the exposure to both melamine alone and combination of melamine and cyanuric acid with the increased of dose (Table 2).

Changes of ER/PR mRNA in ovary of female mice

We investigated the effect of melamine alone or combination with cyanuric acid on the expression of ER-α and PR mRNA in ovary of female mice using real-time PCR technique. The obtained results were presented in Figure 3. With the increasing of dosage, the animals treated with melamine alone with different concentration (2 mg/kg/, 10 mg/kg, 50 mg/kg, respectively) had a decreasing tendency in the relative expression of ER-α mRNA (Figure 3a). In comparison to control group, the animals treated with high dose of melamine alone (50 mg/kg) showed a significant decrease in relative expression of ER-α mRNA ($P < 0.05$). Although the animals from the mixture group of melamine and cyanuric acid also exhibited similar tendency to those treated with melamine alone, less abundance of ER-α mRNA was observed in the animals from co-administration group (Figure 3a). Compared with the control group, the relative expression of ER-α mRNA exhibited a significant decrease in mixture group of melamine and cyanuric acid with low dose (1 mg/kg, $P < 0.05$), middle dose (2 mg/kg, $P < 0.01$), and high dose (1 mg/kg, $P < 0.01$). The results on relative expression of PR mRNA were presented in Figure 3b. As shown, the change pattern of PR mRNA in the animals treated with melamine alone or combination of melamine and cyanuric acid were high similar to those of ER mRNA. In comparison to control group, however, no significant difference was observed in relative expression of PR mRNA of co-administration group of melamine and cyanuric acid with low dose (1 mg/kg $P < 0.01$) (Figure 3b).

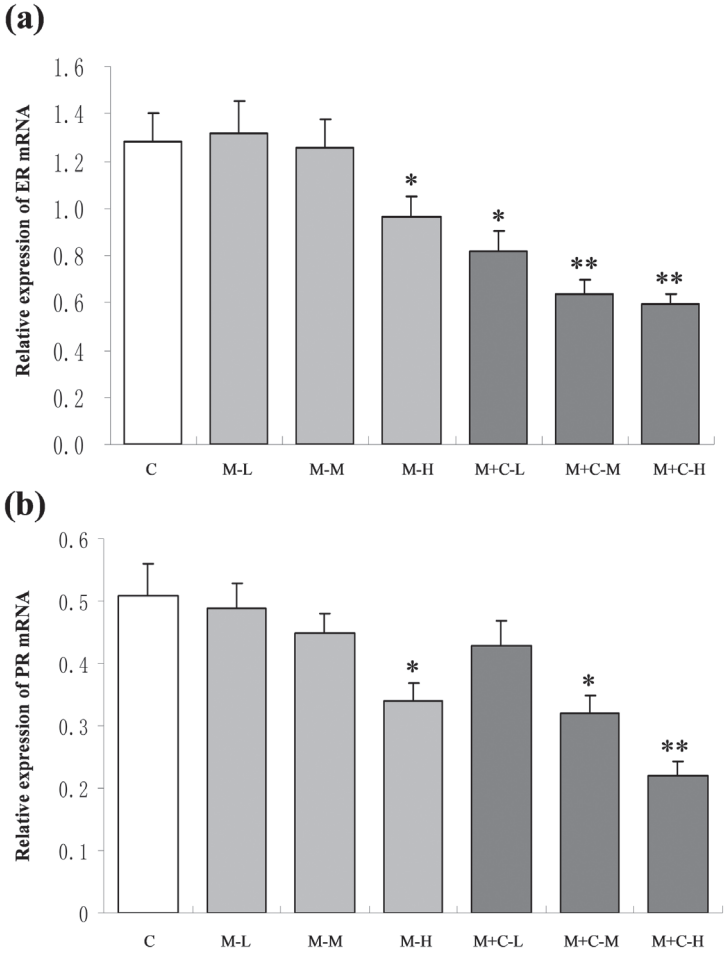

Figure 3: The effect of melamine alone or combination with cyanuric acid on the relative expression of ER and PR mRNA in ovary of female mice. (a) Expression changes of ER mRNA in ovary of female mice. (b) Expression changes of ER mRNA in ovary of female mice. The expression abundance of ER and PR mRNA were normalized against the internal control gene β-actin. Bars represent the means ± standard error. In comparison to control group, * standing for significant difference ($P < 0.05$), and ** standing for significant difference ($P < 0.01$). C = Control group, M-L = Melamine low dosage group (2 mg/kg), M-M = Melamine middle dosage group (10 mg/kg), M-H = Melamine high dosage group (50 mg/kg), M + C - L = Mixture low dosage group of melamine and cyanuric acid (each at 1 mg/kg), M + C - M = Mixture middle dosage group of melamine and cyanuric acid (each at 5 mg/kg), and M + C - H = Mixture high dosage group of melamine and cyanuric acid (each at 25 mg/kg).

DISCUSSION

Melamine alone was generally considered to be of low acute toxicity in mammals (6, 7, 25). It was recorded that melamine was also attempted as a potential anti-cancer agent in the 1960's and 1970's, but was afterwards discarded due to its lack of efficacy (26). However, studies have demonstrated that the combination of melamine with cyanuric acid can cause crystalluria, kidney stones and nephrotoxicity in animals (8, 27-29). Thus, investigations of melamine-related toxicity have mainly focused on the melamine-induced lesions to kidneys. However, the reproductive toxicity of melamine has been less extensively investigated, although it was reported that melamine can pass through the blood-testes barrier, and cause infertility and fetal toxicity (30-32). Until recently, several studies demonstrated that exposure to melamine alone or combination with cyanuric acid can lead to the DNA damage and deformity of sperm (11, 14), Moreover, pathological changes were observed in the testes of mice orally administered with melamine alone or combination with cyanuric acid combination (14, 17). These results suggested that exposure to melamine alone or combination with cyanuric acid can lead to damage to testes in mice and that the toxicity of melamine appears to have been underestimated.

Apoptosis is a gene-regulated process that can be induced by many chemical agents (33, 34). In a recent study, we demonstrated that, in male mice, the ingestion of melamine alone or combination with cyanuric acid can lead to an increase in apoptotic index of spermatogenic cells in a dose-dependent manner (14), but it is unknown if the increase in apoptotic index noted is a direct or indirect effect. In the present study, we investigated the effect of melamine alone or combination with cyanuric acid on expression of 2 crucial genes participating in apoptotic process, namely *Bax* (an apoptosis promoter) and *Bcl-2* (an apoptosis inhibitor). Our data indicated that, within the dose range used, exposure to melamine alone or combination with cyanuric acid promoted the expression of Bax protein, and suppressed the expression of Bcl-2 protein in testis of male mice in a dose-dependent manner (Table 2). These results are generally consistent with those reported recently by Hua *et al.* (2012) (8), and supported the previous findings that ingestion of melamine alone or in combination with cyanuric acid can induce apoptosis in the mice testes (8, 14). Also, our results were similar to those reported that melamine can induce apoptosis in differentiated PC12 cells and preneoplastic urothelial cells (9, 35).

During early apoptosis, Bax is inserted into the mitochondrial membrane and increases the permeability of membrane, ultimately leading to apoptotic cell death of the cell (19, 20). Bcl-2 protein on the other hand prevents this process through preserving mitochondrial integrity (21). Therefore the expression balance between Bax and Bcl-2 is pivotal to the induction of apoptosis. It has been demonstrated that where the ratio of Bax/Bcl-2 is higher than 1, the cells are sensitive to proapoptotic agents (36). In the present study, our results indicated that the ratio of Bax/Bcl-2 increases in groups of both melamine alone and the combination groups of melamine and cyanuric acid in a dose-dependent manner (Table 2). Thus, it might be suggested that the testes cells of male mice are sensitive to melamine alone or the combination with cyanuric acid, and Bax and Bcl-2 may play critical roles in the melamine-induced apoptosis of testes cells in male mice (14). These data from this study are providing insight into the molecular mechanisms underlying the melamine-induced apoptosis in the testes of male mice.

There are two ER subtypes including ER-α and ER-β, which are encoded by *ESR1* and *ESR2* genes, respectively. On the whole, ER-α is considered to be the main receptor for estrogen (37). Estrogen acts through ER-α at the hypothalamus-hypophysis-ovary (HPO) axis to stimulate the release of gonadotrophins, and ultimately plays a role in the regulation of folliculogenesis (38). Studies have demonstrated that female ER-α knockout mice had the defects of anovulation and infertility, which suggested the importance of ER-α in the reproductive system in female mice (23). In the present study, the relative expression of ER-α in ovary was significantly down-regulated in the animals from melamine high dose group (50 mg/kg), as well as mixture groups of melamine and cyanuric acid at low (1 mg/kg), middle (5 mg/kg) and high doses (25 mg/kg) (Figure 3a). These results suggested that melamine might be toxic to the female reproductive system of mice through changing the expression level of ER-α in the ovary, especially in the presence of cyanuric acid.

PR is the member of nuclear receptor superfamily, and is thought to act as transcriptional factor that regulate gene expression by interacting with cognate DNA sequences. Also, it plays a critical role in the regulation of reproductive physiolo-

gy (25). It was reported that PR knockout mice are incapable of undergoing ovulation, even in response to gonadotropin challenge, further indicating that PR is necessary for ovulation (39, 40). In the present study, melamine caused significant decrease in the relative expression of PR in ovary of the female mice administrated with melamine alone (50 mg/kg), the combination of cyanuric acid (each at 5 or 25 mg/kg) (Fig. 3b) compared with the mice from control group. Therefore, it can be suggested that melamine might cause certain toxic effects to the ovulation process of female mice.

In conclusion, the results from the present study indicated that the ingestion of melamine alone or combination with cyanuric acid melamine can promote the expression of Bax protein, but suppress the expression of Bcl-2 protein in testis of male mice. On the other hand, melamine can cause the decrease of expression level of ER-α and PR in ovary of female mice, especially in the presence of cyanuric acid. These results from the present study might be useful in evaluating the melamine-related reproductive toxicity in mammals. Also, they would contribute to the existing toxic profile of melamine.

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