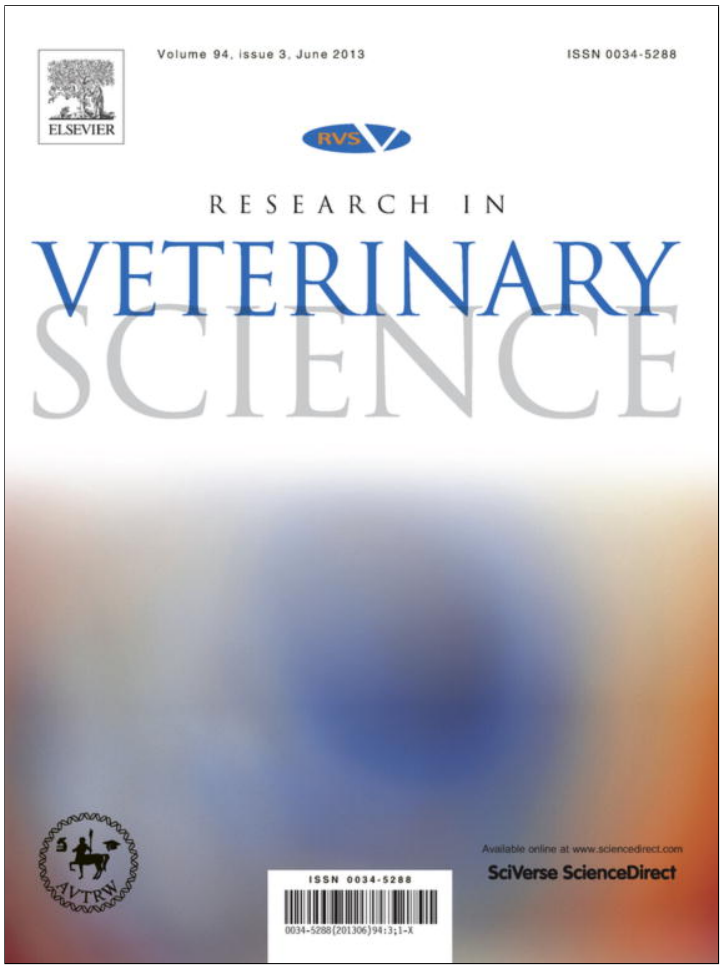

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The reproductive toxicity of melamine in the absence and presence of cyanuric acid in male mice

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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.

2.6. Detection of sperm abnormality

To evaluate sperm abnormality, the right epididymis collected was minced in petri dish containing 2 mL physiological saline with small scissors. And then, the sample was left at 37 °C for 10 min to release sperm. The suspension in medium of physiological saline was filtered using lens cleaning paper, and the filtrate obtained was smeared on clean, grease-free microscope slides. Smears were air-dried, subsequently fixed with methanol. After air-dried, the slides were stained 2% aqueous eosin solution for 1 h, and washed gently with distilled water. One thousand sperms from each mouse were evaluated using a light microscope with an oil immersion objective lens. The spermatozoa, which appeared as banana shaped head, no head, double head, neck torsion, amorphous head, no hook, fold in middle and tail, fat head, and coiled tail, etc. were considered as abnormal. We recorded the types of abnormal spermatozoa, and calculated the sperm abnormality rate as percentages.

2.7. Statistical analysis

Using SAS 6.12 software (SAS Institute, Cary, NC, USA), the statistical comparison were performed with single factor analysis of variance (ANOVA). The data, calculated as a percentage, were expressed as the mean ± S.E. (standard error), and p-value less than 0.05 were considered to be significant.

3. Results

3.1. Clinical observations

In the mice used for investigating the histopathologic changes and apoptosis of spermatogenic cells of testis, no deaths were found in the melamine groups with low (2 mg/kg/day), middle (10 mg/kg/day) and high (50 mg/kg/day) doses, as well as, co-administration groups of melamine and cyanuric acid with low (each at 1 mg/kg/day) and middle (each at 5 mg/kg/day) doses. However, 3 mice from co-administration groups of melamine and cyanuric acid with high dose (each at 25 mg/kg/day) died at days 8, 10 and 13, respectively, and they had exhibited anorexia, dull hair coat and depression before death. Compared with the control group, in the co-administration group of melamine and cyanuric acid with high dose (each at 25 mg/kg/day), significant decrease in body weight was observed on days 8 and 14, whereas there were no significant differences in body weight between control group and the remaining treated groups with melamine alone or the combination of melamine and cyanuric acid (data not shown). At the end of the experiment, the kidneys of the survival mice were grossly enlarged and pale yellow. No obvious changes were noted in the other organs including testis.

In the mice used for evaluating the sperm abnormality, no deaths were found in the treated groups with melamine alone, and the mice treated with high dosage of melamine (1648 mg/kg/day) had a lower body weight versus the negative control groups at the end of the experiment, but without statistical significance. However, all mice administrated with combination of melamine and cyanuric acid (each at 206, 412, or 824 mg/kg/day) died before day 6, and they exhibited anorexia, decreased activity and a hunched posture before death.

3.2. Testicular histopathology examination in mice

In light microscopic examinations, the testis sections of histopathological changes were shown in Fig. 1. The animals from control group presented a normal histological pattern (Fig. 1A). There were 5–8 layers of epithelial cell in seminiferous tubules. Spermatogenic cells at all stages exhibited normal development including spermatogonia, primary spermatocytes, secondary spermatocytes, and sperm cells, and were arranged in a well defined pattern, as well as, the spermatids were embedded in sertoli cells. The animals from melamine-treated group with low dose (2 mg/kg/day) exhibited had irregular nucleus shape in some cells (Fig. 1B), but no other obvious change was found by light microscope observation. Seminiferous tubules from melamine-treated group with middle dose (10 mg/kg/day) had indistinct basement membrane, and spermatogenic cells at all stages exhibited a slightly loosened organization with decreased cell layers. Also, irregular nucleus shape with intense staining was observed in a few cells from primary spermatocytes, secondary spermatocytes and sperm cell showed (Fig. 1C).

Damage of basement membrane of seminiferous epithelium in seminiferous tubules was observed in the animals from melamine-treated groups with high dosage (50 mg/kg/day), with decreased layers of epithelial cell and the structural damage was found in many spermatogenic cells at all stages. Also, swelling and lysis of nucleus occurs in the primary spermatocytes, secondary spermatocytes and the sperm cells with the uneven size and staining of varying intensity (Fig. 1D). In addition, a reduction in the number of sperm or even no mature sperm was observed in some sertoli cells of seminiferous tubule.

Co-administration of melamine and cyanuric acid with low dose (each at 1 mg/kg/day) caused the irregular arrangement of some nuclei in shape (Fig. 1E). In the group of co-administration of melamine and cyanuric acid with middle dose (each at 5 mg/kg/day), the layers of epithelial cells were indistinct, and a reduction in the number of primary spermatocytes, secondary spermatocytes and spermatids was observed. Also, spermatocytes exhibited the swelling and friability of nuclei with an uneven staining (Fig. 1F). Co-administration of melamine and cyanuric acid with high dose (each at 25 mg/kg/day) caused the reduction of spermatogenic cell layer with a loose organization. On the other hand, swelling and lysis of nucleus were revealed in spermatogenic cells with staining of varying intensity (Fig. 1G), and a reduction in the number of sperm cells (or even no mature sperm) was observed in some seminiferous tubule.

3.3. Ultrastructural changes of testicular tissue in mouse

In electron microscope examinations, compared with control group, no obvious change was observed in the leydig cells and seminiferous cells at all stages of the mice from melamine group with low dose (2 mg/kg/day), but the mice from middle dose group of melamine (10 mg/kg/day) exhibited margical collection of heterochromatins with abnormal nuclear shape in interstitial cells, and dilatation of rough endoplasmic reticulum was noted in spermatogonia and primary spermatocytes.

High dose group of melamine (50 mg/kg/day) resulted in the obvious changes in interstitial cells, including irregular shape of nucleus, margical collection of heterochromatins, presence of more mitochondria, ribosome and lipid droplet in intracytoplasm (Fig. 2A). Also, microvilli were visible on the surface of many cells. Spermatogonial cells exhibited intense staining, and indistinct nuclear membrane. Lysis of kytoplasm was also noted in some spermatogonial cells (Fig. 2B). Both primary spermatocytes and secondary spermatocytes showed indistinct nucleus membrane, more mitochondria in intracytoplasm with a presence of dilated rough endoplasmic reticulum in part of the cells (Fig. 2C). Additionally, the sperm cells had hyperchromatic acrosomal granules in acrosomal vacuoles, and the dilatation of rough endoplasmic reticulum was also observed in the some sperm cells (Fig. 2D).

In comparison to control group, in co-administration groups of melamine and cyanuric acid with low (each at 1 mg/kg/day) and middle (each at 5 mg/kg/day) doses, irregular nucleus shapes were

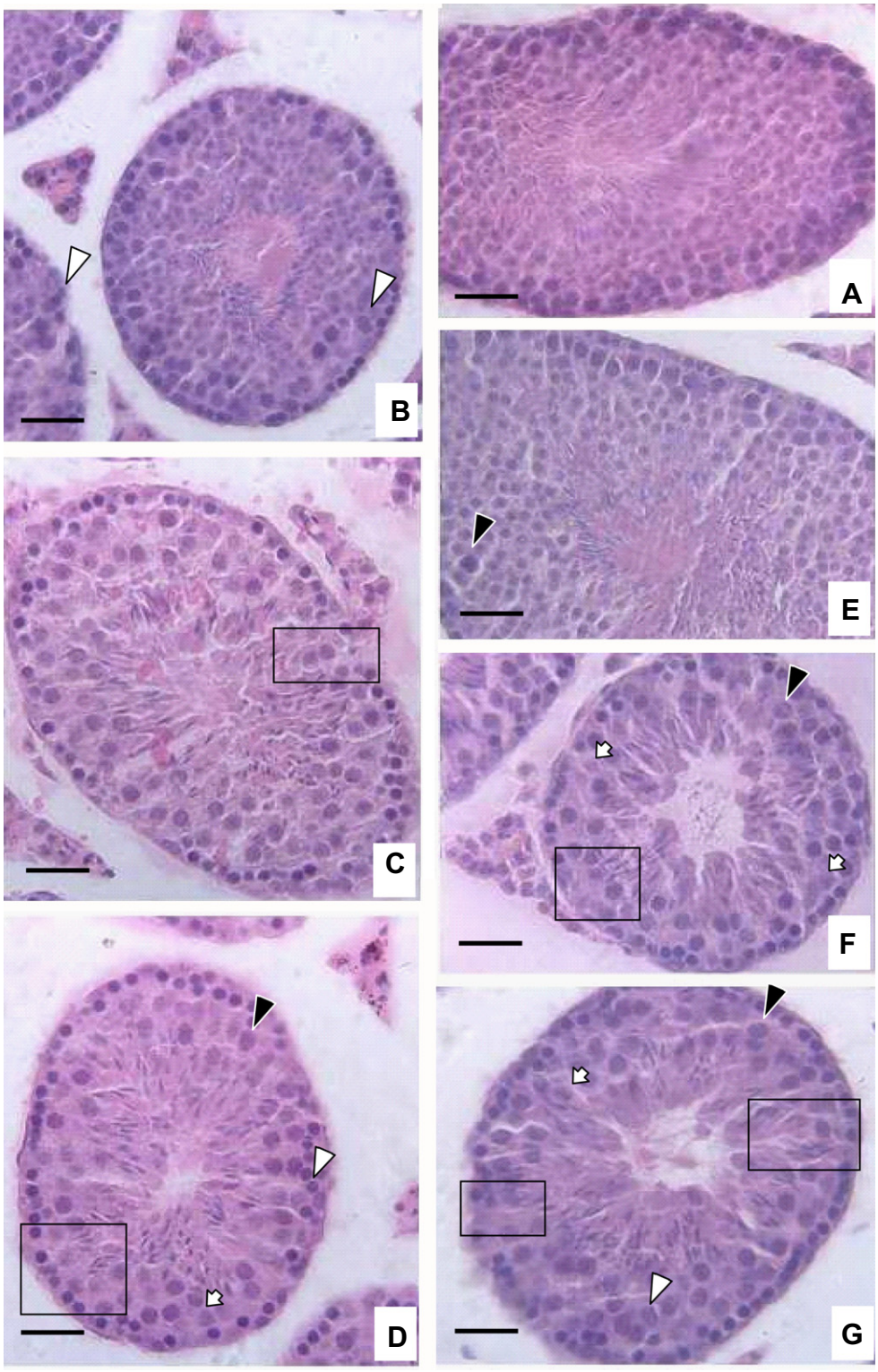

Fig. 1. Changes in testicular sections of mouse. (A) control group; (B) melamine-treated group with low dose (2 mg/kg/day); (C) melamine-treated groups with middle dose (10 mg/kg/day); (D) melamine-treated groups with high dose (50 mg/kg/day); (E) co-administration of melamine and cyanuric acid with low dose (1 mg/kg/day of each); (F) co-administration of melamine and cyanuric acid with middle dosage (5 mg/kg/day of each); (G) co-administration of melamine and cyanuric acid with high dose (25 mg/kg/day of each). The irregular nucleus shapes were indicated by the white triangles. The swelling of nuclei was indicated by the black triangles. The lysis of nuclei was indicated by the large white arrows. The spermatogenic cells, which had a loose organization with decreased layers, were boxed with black line. Bars = 30 μm.

observed in interstitial cells with margical collection of heterochromatins in a few cell nuclei. Also, the mice from co-administration groups of melamine and cyanuric acid with middle doses (each at 5 mg/kg/day) exhibited some indistinct of nuclear

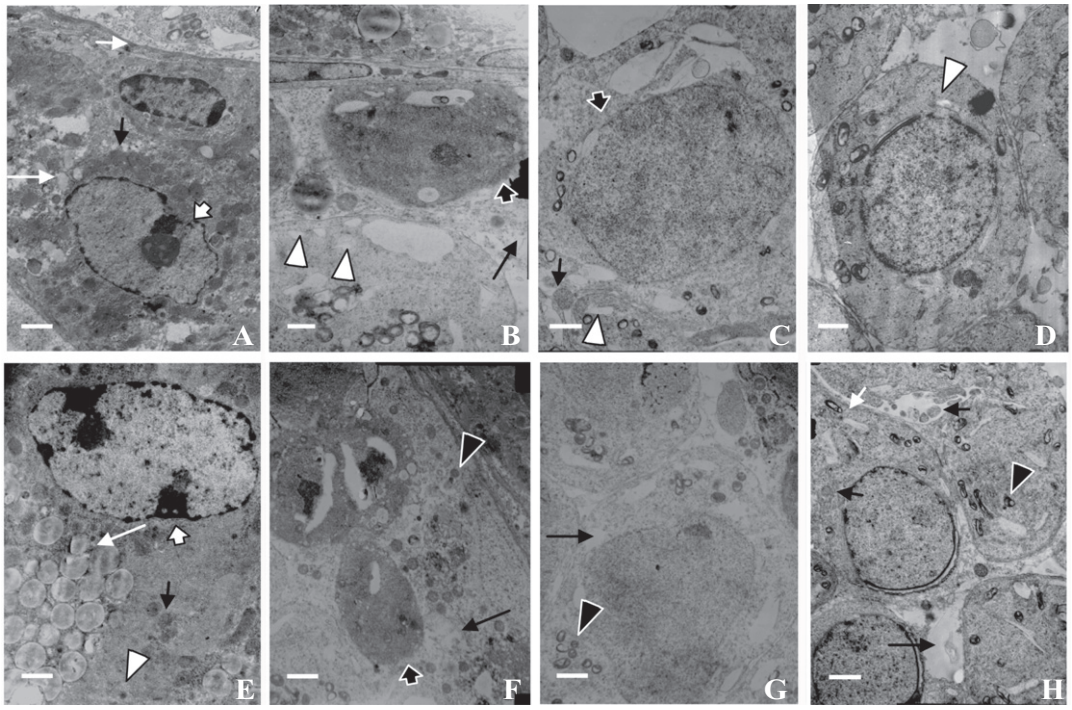

Fig. 2. Ultrastructural changes of testicular tissue in mouse. A and E represented the testicular leydig and interstitial cells, respectively. B and F represented spermatogonial cells. C and G represented the primary spermatocytes. D and H represented sperm cells. Irregular shapes of nucleus and margical collections of heterochromatins were indicated by large white arrows. Presences of more mitochondria, ribosome and lipid droplet were indicated by short black line arrows, short white line arrows, and long white line arrows, respectively. The intense staining and indistinct nuclear membrane in spermatogonial cells were indicated by large black arrows. Lysis of kytoplasm of some spermatogonial cells was indicated by long black line arrows. The dilatation of rough endoplasmic reticulum was indicated by white triangles. Lysis of nuclear membrane was indicated by large black arrows. Lysosomes were indicated by black triangles. Bars = 1 μm.

membrane, and lysis of cytoplasm in spermatogonia and primary spermatocytes. In addition, we also observed the lysis of cytoplasm in sperm cells.

Co-administration of melamine and cyanuric acid with high dose (each at 25 mg/kg/day) resulted in profound pathological damage to testis of male mice. Interstitial cell exhibited irregularly shaped nuclei with margical collection of heterochromatins, and many lipid droplets, mitochondria, and lysosomes were observed in intracytoplasm (Fig. 2E). Near the basement membrane, there were spermatogonial cells exhibiting the lysis of nuclear membrane, intense staining of kytoplasm, and cytoplasmic lysis with many cytolysosomes in plasmolysis (Fig. 2F). Nuclei of primary spermatocytes became bigger in size with indistinct nucleus membrane and lysis of some cytoplasm (Fig. 2G). Secondary spermatocytes showed the similarities to that of primary spermatocytes but having more mitochondria and ribosome in cytoplasm. Stained intensely acrosome granules were observed in acrosome vesicle of sperm cells. There were many mitochondria, ribosomes and cytolysosomes in intracytoplasm, and the lysis of intracytoplasm also was observed in a small amount of sperm cells (Fig. 2H).

3.4. Apoptosis examination of spermatogenic cells

As observed from Fig. 3, the apoptotic nuclei of positive cells on convoluted seminiferous tubule were stained as brown in light microscopic examinations. In spermatogenic cells of testis, positive cells stained were observed in all mice from different experiment groups, which were different only in number. With the increasing of dosage, the animals treated with melamine alone with different concentration (2 mg/kg/day, 10 mg/kg/day and 50 mg/kg/day, respectively) exhibited an increasing tendency in apoptotic index of spermatogenic cells (Fig. 4). Compared with control group, the animals treated with high dose of melamine alone (50 mg/kg/day) had a significant increase in apoptotic index of spermatogenic cells ($p < 0.05$). Although co-administration of melamine and cyanuric acid also showed similar tendency to administration group of melamine alone in apoptotic index, more severe apoptosis was observed in both middle (each at 5 mg/kg/day) and high dose (each at 25 mg/kg/day) groups (Fig. 3E–G). As showed in Fig. 4, in comparison to the control group, the apoptotic index had a significant increase in co-administration group of melamine and cyanuric acid with both middle dose (each at 5 mg/kg/day, $p < 0.05$) and high dose (each at 25 mg/kg/day, $p < 0.01$).

3.5. Detection of sperm abnormality rate

As shown in Table 1, it is obvious that treating male mice with melamine alone resulted in profound altered sperm morphology. These abnormalities included: banana shaped head (Fig. 5b), no head (Fig. 5c), double head (Fig. 5d), neck torsion (Fig. 5e), no hook (Fig. 5f), amorphous head (Fig. 5g), fold in middle and tail (Fig. 5h), fat head (Fig. 5i), and coiled tail (Fig. 5j). Unlike the mice from negative control group, in which only 1.93% of the epididymal sperms exhibited abnormal morphology, melamine alone treated mice with different doses (412, 824, and1648 mg/kg/day) had 2.31%, 2.83% and 5.63% abnormality rate of sperm, respectively (Table 1). Compared with negative control group, all melamine alone treated groups, were significantly higher in abnormality rate of sperm ($p < 0.05$ or $p < 0.01$; Table 1), suggested that melamine alone can cause abnormal sperm morphology. 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 was obtained on sperm abnormality.

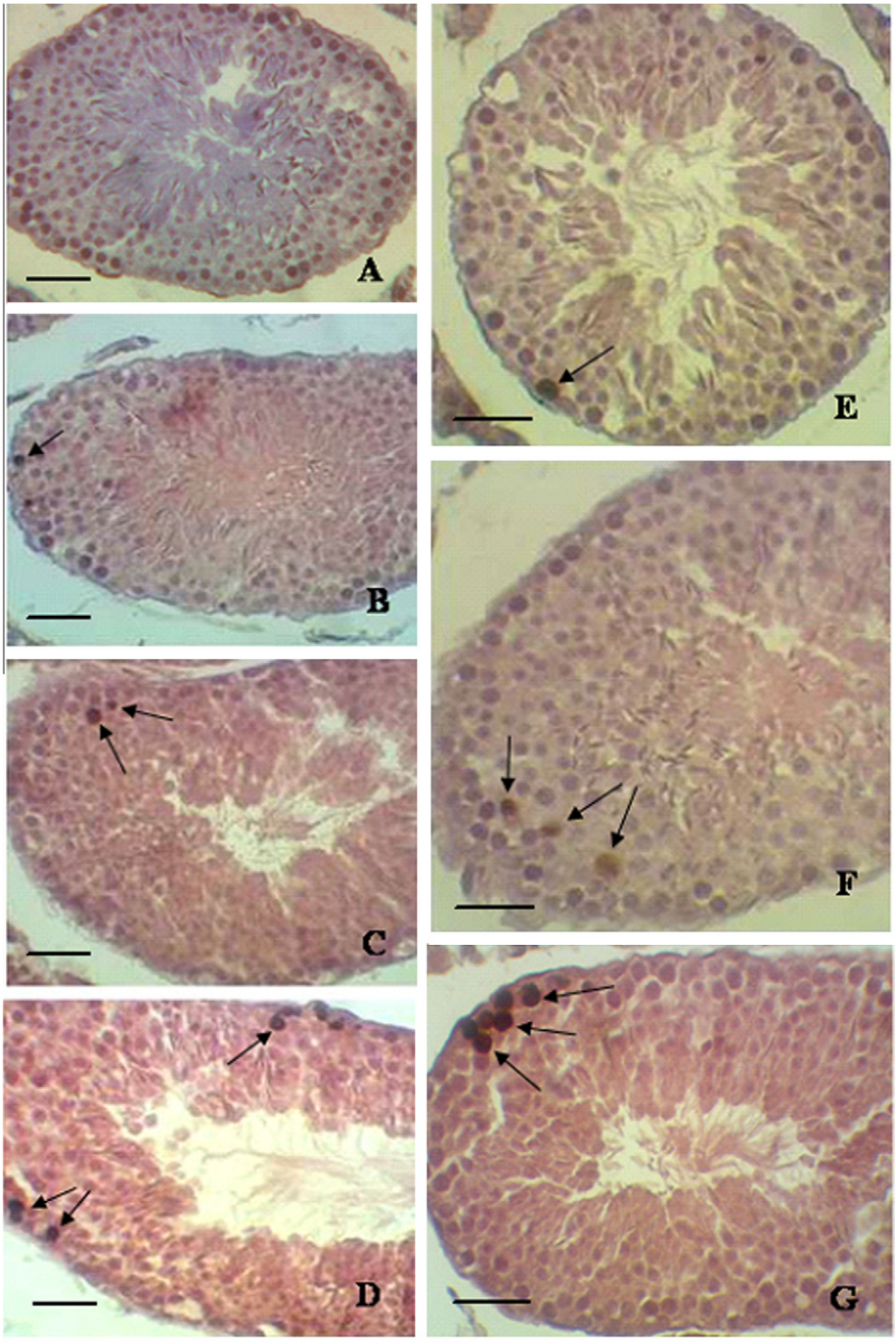

Fig. 3. Testicular section from different treated groups shows apoptosis of spermatogenic cells that were detected by TUNEL assay. (A): control group; (B) melamine low dose group (2 mg/kg/day); (C): melamine middle dose groups (10 mg/kg/day); (D): Melamine high dose group (50 mg/kg/day); (E): mix low dose group of melamine and cyanuric acid (1 mg/kg/day of each); (F): mix middle dose group of melamine and cyanuric acid (5 mg/kg/day of each); (G): mix high dose group of melamine and cyanuric acid (25 mg/kg/day of each). The apoptotic nuclei of positive cells were indicated by black arrows. Bars = 30 μm.

4. Discussion

Melamine was used widely in the manufacture of amino resins and plastics (Cook et al., 2005). In the 1960s and 1970s, melamine was also explored as potential anti-cancer agents, but was afterwards abandoned considering its toxicity and insufficient benefit (Langman et al. 2009).Toxicity data of melamine mainly come from studies in cat, dog (Brown et al., 2007; Puschner et al., 2007), mouse (Zhang et al., 2011), rat (Chen et al., 2009; Pang et al., 2011), pig and fish (Reimschuessel et al., 2008, 2009). The most common toxicity is renal toxicity, which is also the area of most concern to nephrologists (Hau et al., 2009). Thus, previous investigations have focused

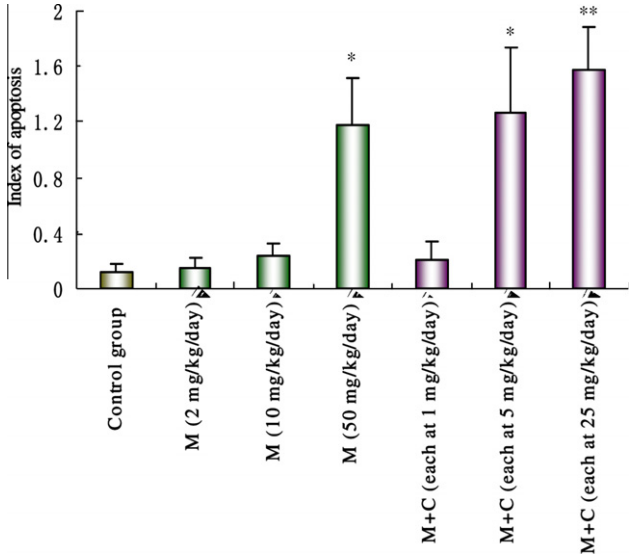

Fig. 4. The effects of melamine in the presence and absence of cyanuric acid on apoptosis index of spermatogenic cells in testes of male mice. In comparison to control group, * indicated significant differences ($P < 0.05$), and ** indicated significant differences ($P < 0.01$). M = Melamine, and M+C = The combination of melamine and cyanuric acid.

on the effect of melamine-induced crystalluria, kidney stones and nephrotoxicity, but there has been less extensively investigation about the toxicity of melamine to other tissues and organs. Recent studies demonstrated that melamine could be found in central nervous system, such as cortex, striatum, hippocampus, cerebellum, and brain stem (Wu et al., 2009; Wang et al., 2011), and effectively inhibit outward potassium currents and delay rectified potassium current in a concentration-dependent manner in rat hippocampal CA1 neurons (Yang et al. 2010). More recently, an investigation by Han et al. (2011) indicated that melamine could inhibit differentiated PC12 cell proliferation by inducing apoptosis, and oxidative stress was also present in the process of apoptosis. Also, Wang et al. (2011) demonstrated that that melamine alone, even at low levels, could affect the morphology and caspase-3 activity of hippocampal neurons. The results from these studies suggested that melamine is likely to have potential toxicity on other tissues and organs besides kidney.

Histopathological evaluations are commonly used methods for detecting organ-specific effects related to chemical exposure (Travlos et al., 1996; Crissman et al., 2004). In the present work, our data demonstrated that melamine caused certain pathological injury (Fig. 1B–D). For example, the damage of basement membrane of seminiferous epithelium in seminiferous tubules was observed by light microscopic examinations in this study (Fig. 1D). The basement membrane was reported to be important in maintaining the structural and functional intergrity of tissues, and any structural changes in basement membrane are associated with severe functional impairment of the testis (Richardson et al., 1998; Mahmoud, 2012). On the other hand, it is also stated that the disruption of the seminiferous epithelium was indicative of male reproductive risk (Heidari et al., 2012). Therefore, our results suggested a gonadotoxic potential of melamine to male mice.

In this study, additionally, a reduction in the amount of sperm or even no mature sperm was observed in some sertoli cells. Spermatogenesis occurs in several stages and destruction of it in any stage can affect the whole process (Yavasoglu et al., 2008). In the light of the results from this study, we speculated that the structural damage in spermatogenic cells at different stages in melamine treated group (50 mg/kg/day) might cause the reduction in the amount of sperm.

In the present work, we also observed the ultrastructural changes in testis of male mice treated with different doses of melamine (Fig. 2B–D) in a dose-dependent manner, which further suggested that melamine have certain toxicity on testis. Hau et al. (2009) also suggested that toxicity of melamine may not be limited to renal stone formation in animal studies if melamine is present in high dosages or in combination with cyanuric acid. Here, we firstly showed the reproductive toxicity of melamine on male mice. Although melamine is generally thought to be low toxicity according to the previous studies, the toxicity of melamine appears to be underestimated. Therefore, a larger investigation of toxic effect of melamine on reproductive system should be performed for a more complete toxic profile of melamine.

Cyanuric acid is structurally related to melamine, and it itself appears to be of rather low acute toxicities according to the limited toxicological data that has been published for this compound (Puschner et al., 2007). Hodge et al. (1965) demonstrated that the administration of 8% sodium cyanurate in the diet of rats (for 20 weeks) and dogs (for 16–24 months) resulted in histologic changes in the kidneys consisting of dilatation of the distal collecting tubules and ducts of Bellini with focal areas of epithelial proliferation. Also, the subchronic feeding of sodium cyanurate at up 700 mg/kg and 2200 mg/kg, in rats and mice, respectively, resulted in bladder calculi and some associated bladder epithelial changes, and no other adverse effects were observed (Hammond et al., 1986).

However, studies have demonstrated that cyanuric acid can interact with melamine (Puschner et al., 2007), and cyanuric acid combined with melamine leads to the alteration of the kinetic characteristics of melamine (Pang et al., 2011). Co-administration with melamine and cyanuric acid resulted in acute renal failure in cats within 48 h after in gestion (Hau et al., 2009). Recently, several studies also showed that the combination of melamine and cyanuric acid is responsible for renal impairment in some other species, such as dog (Burns, 2007), rat (Pang et al., 2011), pig and fish (Reimschuessel et al., 2008). Thus, the co-administration of melamine and cyanuric acid are known to have nephrotoxicity, but little information is available about combined effects of melamine and cyanuric acid to other tissues and organs. Recently, the combined toxicity of melamine and cyanuric acid on liver was investigated by Xie et al. (2011) in mice, demonstrating that the liver damage induced by mixture of melamine and cyanuric acid exhibited an obvious dose-effect relationship. Based on the results obtained, the authors speculated that the liver damage was likely to result from the apoptosis of liver cells where the genes of Bax and Caspase-3 were involved. Therefore, it was suggested that the combined effects of melamine and cyanuric acid was not limited to the kidney. On the other hand, several recent investigations demonstrated that renal failure resulting from the combined exposure to melamine and cyanuric acid caused major changes in other organs besides kidney and altered the body's normal hemodynamics, such as in rats (Jacob et al. 2012), catfish and rainbow trout (Reimschuessel et al., 2010a,b). Taken together with our results, it was suggested that renal failure was likely the cause of any effects noted in the testis of mice treated with melamine and cyanuric acid.

It is well known that apoptosis of spermatogenic cells is essential for the normal maintenance of spermatogenesis, but a relatively increase in the apoptosis of spermatogenic cells might cause defective spermatogenesis resulting in infertility (Moline et al., 2000). Increases in the incidence of testicular apoptosis are often observed as a result of various forms of physical or chemical injury to the testis (Richburg, 2000). The TUNEL technique provides a useful general screen for cell death (Weber et al., 2004). Based on the TUNEL assay, in this study, the mice treated with high dose of melamine (50 mg/kg/day) had a significant increase in apoptotic

Table 1
Effect of melamine in present and absent of cyanuric acid on sperm abnormality in male mice.

Groups	Size of group (n)	Number of sperm observed	Tteratosperm number of each type									Total number of teratosperm	Rate of teratosperm (%)
			Banana shaped head	No head	Double head	Neck torsion	Amorphous head	No hook	Fold in middle and tail	Fat head	Coiled tail		
Negative control	8	8000	4	2	5	22	24	23	54	7	13	154	1.93 ± 0.34
Melamine (412 mg/kg/day)	8	8000	6	3	8	16	32	19	67	9	25	185	2.31 ± 0.47[a]
Melamine (824 mg/kg/day)	8	8000	7	13	6	36	45	31	120	14	34	306	3.83 ± 0.39[b]
Melamine (1648 mg/kg/day)	8	8000	16	18	27	63	44	47	180	13	48	450	5.63 ± 0.58[b]
Positive control	8	8000	29	26	22	92	71	61	207	34	51	583	7.29 ± 0.47[b]

[a] Standing for significant difference compared with negative group, $p < 0.05$.
[b] Standing for significant difference compared with the negative control group, $p < 0.01$.

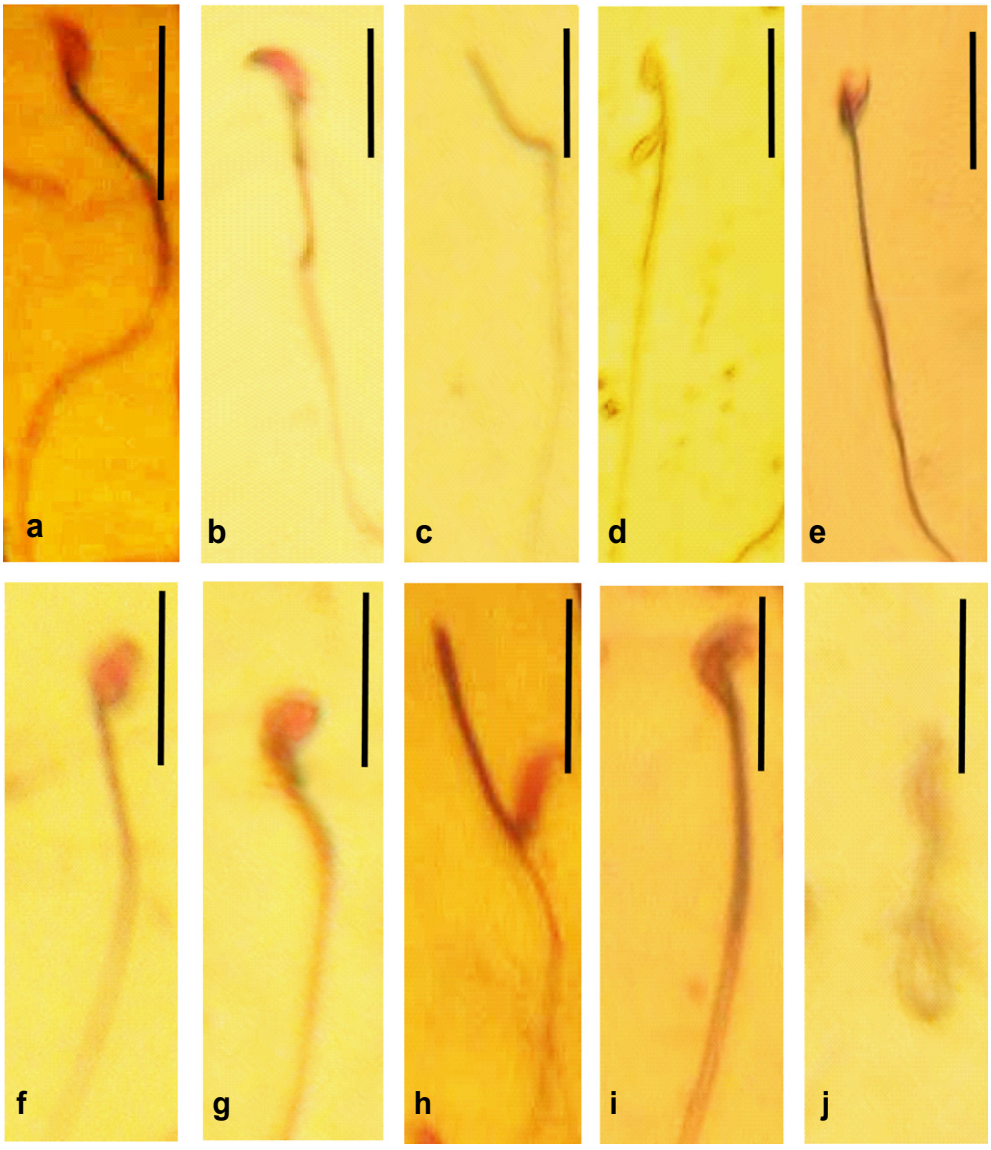

Fig. 5. Photomicrographs of epididymal sperm smears stained with aqueous eosin. Normal sperm (a) and abnormal sperm (b–j): (b) banana shaped head; (c) no head; (d) double head; (e) neck torsion; (f) no hook; (g) amorphous head; (h) fold in middle and tail; (i) fat head; (j) coiled tail. Bars = 20 μm.

index of spermatogenic cells ($p < 0.05$) compared with the control group, which suggested that high concentration of melamine not only was able to induce apoptosis of spermatogenic cells in male mice, but also might be a risk factor for tesitis toxicity. We also observed a low incidence of spontaneous apoptosis in normal mice testis from the control group (Fig. 3A), which was consistent with the fact that apoptosis of spermatogenic cells is essential for the normal maintenance of spermatogenesis (Moline et al., 2000).

On the other hand, compared with 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 (each at 25 mg/kg/day) doses (Fig. 4). Pang et al. (2011) demonstrated that, in comparison to melamine administered alone, co-administration with cyanuric acid reduced and slowed down the uptake of melamine into blood systematic circulation and the elimination of melamine. In fact, cyanuric acid would not cause directly greater accumulation of melamine in testis, but the effect of renal failure resulting from the combination of melamine and cyanuric acid itself might cause multiple organ failure including testis, which correlated well with the generation and development of testis toxicity. However, further studies would be needed to determine the mechanisms underlying apoptosis of spermatogenic cell in mice, which were induced by the combination of melamine and cyanuric acid.

Sperm is widely used in evaluating the effect of chemicals on offspring deformation resulting from genetic materials by examining the quality of sperm (Zhang et al., 2011). In the present work, we demonstrated that treated male mice with melamine alone in different doses (412, 824, and 1648 mg/kg/day, respectively) exhibited profound altered epididymal sperm morphology, particularly the head abnormality was observed to be most dominant in the abnormality of sperm (Fig. 5, Table 1). Previous studies showed that detached head of sperm is associated with the testicular degeneration (Mahmoud, 2012). Also, it was reported that these deformities might have been caused by the alterations in the pattern of chromatin condensation (Zamboni, 1991; Zamboni, 1992; Pinart et al., 1998). On the other hand, however, a recent investigation conducted by Zhang et al. (2011) demonstrated that melamine has ability to increase sperm abnormality rate, which were confirmed well by our results from this work, as well as, it can cause DNA damage based on the detection through single cell gel electrophoresis. Taken together with our results, it supported the previous postulation that morphological abnormalities of sperm might result from the damage of testicular DNA (Lister and McLean, 1997). However, all mice administrated with combination of melamine and cyanuric acid (each at 206, 412, or 824 mg/kg/day) died before day 6. Therefore, no data on sperm abnormality was obtained from the mice. The mice exhibited anorexia, depression, dull hair coat and weight loss before death. At autopsy, the kidneys of mice were pale yellow color, swollen, and some irregularities also were noted on the surface of the kidney as described in rats by Kobayashi et al. (2010) where the rats were co-administrated with the combination of melamine and cyanuric acid at each 120 mg/kg/day. No obvious changes were noted in the other organs including testis. We speculated that the mice were likely to die of acute renal failure resulting from the combination of melamine and cyanuric acid. Similar deaths have also been observed in rats which were co-administrated with the combination of melamine and cyanuric acid (each at 120 mg/kg/day) (Kobayashi et al., 2010).

In conclusion, the results from the present work indicated that melamine had certain toxic effects on testes of male mice with the increase of sperm abnormality rate, especially when ingested in high concentration. Renal failure from the combined exposure to melamine and cyanuric acid was likely the cause of any effects noted in the testis of mice treated both melamine and cyanuric acid. These results might help us to evaluate the toxicity of melamine on reproductive system of male animal, and they also would be a supplement to the existing toxic profile of melamine. Also, they should be useful in the search of potential toxicity of cyanuric acid.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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