

# Effect of Melamine in the Absence and Presence of Cyanuric Acid on Ultrastructure of Visceral Organs in Male Mice

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## ABSTRACT

Melamine, has been widely used in the production of amino resins and plastics. The combination of melamine and cyanuric acid was thought to be responsible for renal crystal formation and renal damage in animals. In the present work, we investigated the effect of melamine in the absence and presence of cyanuric acid on ultrastructure of multiple visceral organs in mice including kidney, liver, spleen, stomach wall and small intestine. Based on the electron microscopic examination, in comparison to control group, the administration of melamine alone with a dose at 50 mg/kg/day for 14 days caused pathological injury to liver, kidney, spleen, stomach wall and small intestine wall of mice. On the other hand, compared with that of melamine alone (50 mg/kg/day), co-administration of melamine and cyanuric acid (each at dose: 25 mg/kg/day) resulted in more severe pathological changes to the examined visceral organs. These findings suggested that melamine alone or its combination with cyanuric acid appears to be systemically toxic to mice. Moreover, toxic effects of melamine on different visceral organs of mice were further aggravated by the combination with cyanuric acid. These results might be useful in evaluating the melamine-induced pathology in animals.

**Key Words:** Mouse, Pathology, Melamine, Cyanuric acid, Ultrastructural changes.

## INTRODUCTION

Melamine, an organic nitrogenous compound, is widely used in the production of plastics, dyes, glues, dishware, fertilizers, kitchenware and fabrics (1, 2). Since 2008, the occurrence of an outbreak of urinary stones in infants and children consuming melamine-adulterated formula and milk in China, there has been world-wide concern about kidney disease stemming from melamine exposure (3). In animals, it was demonstrated that melamine and cyanuric acid (a byproduct of melamine synthesis) in combination cause crystal formation and subsequent renal failure (4-7). Thus, previous investigations have mainly focused on the effect of melamine-induced crystalluria, kidney stones and nephrotoxicity.

However, it was suggested that toxicity of melamine may not be limited to renal stone formation in animal studies, particularly when melamine is present in high dosages or in combination with cyanuric acid (1). Recent investigations revealed the presence of melamine in the central nervous system of rats, including brain stem striatum, hippocampus, cortex, and cerebellum of rats (8, 9). Also, it was reported that melamine could affect the morphology and caspase-3 activity of hippocampal neurons even at low levels (9), as well as, it could inhibit the proliferation of differentiated PC12 cell by inducing apoptosis with the presence of oxidative stress in the process (10). Another investigation by Xie and colleagues (2011) showed that the co-administration of

melamine and cyanuric acid resulted in the liver damage of mice in an obvious dose-dependent pattern, where the apoptosis of liver cells were involved with expression changes of *Bax*, *cyt-c* and *caspase-3* genes at both the mRNA and protein level (11). Additionally, the potential toxicity of melamine on sperm was also investigated in male mice by Zhang *et al.* (2011), demonstrating that melamine has the ability to increase sperm deformity rate and DNA damage based on the sperm single cell gel electrophoresis, although it has no mutagenic function and does not have the ability to induce malignant cell transformation (12).

To date, however, the detailed morphological effects of melamine in the absence and presence of cyanuric acid on different visceral organs has not been reported by electron microscopy. In addition, there has been little information about the toxic effects of melamine alone or its combination with cyanuric acid on the spleen, stomach, and intestine in mammals.

In the present work, therefore, we investigated the effect of melamine in the absence and presence of cyanuric acid on ultrastructure of multiple visceral organs in mice including kidney, liver, spleen, stomach wall and small intestine by electron microscopy, to supplement the toxicological profile of melamine, and also provide useful information for evaluating the melamine-induced pathology in mammals.

## MATERIALS AND METHODS

### Animals and treatments

The protocol for the present work was approved by the Animal Experimental Committee of Shenyang Agricultural University. Male mice of the Kunming strain (12-week-old, 35-40 g) were provided from Beijing Fukang Biological Technology Co., Ltd. (SCXK 2009-0004, Beijing, China). Kunming mice are an outbred mouse line in China. These animals have been widely used in pharmacological, toxicological, medicinal and biological research and testing due to their high disease resistance, good survival rate, high breeding coefficient and good adaptability.

The animals were SPF grade. After 15 days of acclimation, the mice were divided into three groups (each group, N=10), including a control group, melamine group and combination group of melamine and cyanuric acid. The animals were maintained in controlled laboratory conditions of 12 h dark/light cycle, 22 ± 2 °C temperature, and 35-65% rela-

tive humidity. The mice 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).

The mice from control group were administered by gastric gavage with physiological saline daily. The mice from melamine group were administered with melamine alone by gastric gavage, at doses of 50 mg/kg/day. The mice of combination group were co-administered with melamine and cyanuric acid, each at doses of 25 mg/kg/day. These doses were selected according to the "Procedures and Methods for Toxicological Assessment on Food Safety" compiled by Ministry of Health, P.R. China (13) and the published references by Chen *et al.* (14) and xie *et al.* (11).

All food and water were tested for both melamine and cyanuric acid according to the described method of Heller *et al.* (15), and neither melamine nor cyanuric acid contaminant was detected above the limit of quantitation of the method being 0.5 ppm.

Melamine and cyanuric acid were mixed with water at room temperature, and each dose was mixed just before dosing each animal. All administrations were conducted via gastric gavage for 14 consecutive days. Body weight was monitored on days 4, 9 and 14. All animals were observed daily for any clinical signs during the study period. At the end of the experiment, all surviving mice of each group were sacrificed by cervical dislocation, and the tissue samples were collected immediately from kidney, liver, spleen, stomach and duodenum of each mouse. The samples were fixed in 2.5% glutaraldehyde for 12 h.

### Chemicals and reagents

Melamine (>99%) was obtained commercially from Sinopharm Chemical Reagent Beijing Co., Ltd (Beijing, China). Cyanuric acid (>98%) was purchased from Shanghai Crystal Pure Industrial Co., Ltd (Shanghai, China). Other chemicals were analytical grade including glutaraldehyde, alcohol, epoxy resin, osmium tetroxide, uranyl acetate and lead citrate obtained from the reagent branch of Shenyang Agricultural University.

### Electron microscopy examination

The tissues from different visceral organs including kidney, liver, spleen, stomach and intestine were cut into sections of

approximate 1 mm<sup>3</sup>. After rinsing with PBS, the specimens were fixed in 1% osmium tetroxide for 2 h. They were then processed with standard dehydration in graded ethanol for 10 min. Subsequently, the specimens were embedded in epoxy resin. Ultrathin sections were cut using a diatome diamond knife. For electron microscopy examination, the specimens were stained with uranyl acetate and lead citrate for 10 min, respectively. We observed the ultrathin sections using a JEM-1010 transmission electron microscope (JEOL, Tokyo, Japan).

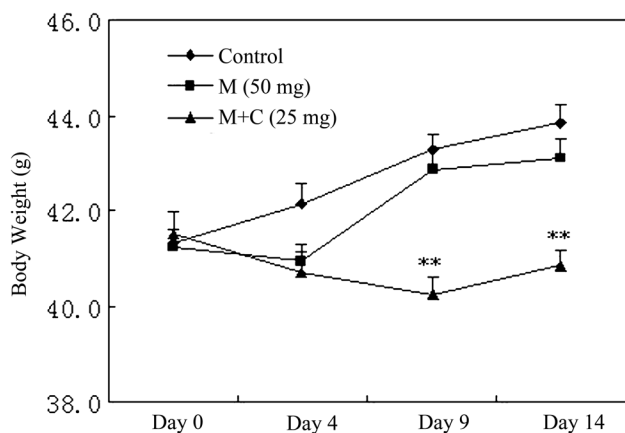
### Statistical analysis

Body weights were compared using the Student's t-test. Probability values of less than  $p < 0.05$  were considered significant.

## RESULTS

### Clinical observations

No deaths were encountered in the control or melamine group (50 mg/kg/day), however, three mice from co-administration groups of melamine and cyanuric acid (each at 25 mg/kg/day) died at days 9, 10 and 12, respectively. They exhibited anorexia, dull hair coat, decreased activity, depression and a hunched posture before death. In comparison to the mice from control group, significant decrease in body weight ( $p < 0.01$ ) was observed in the mice from co-administration groups of melamine and cyanuric acid (each at 25 mg/kg/day) on days 9 and 14, whereas there were no signifi-



**Figure 1:** The changes in body weight of the mice from different groups which were monitored on days 4, 9 and 14. M = Melamine (50 mg/kg/day), and M+C = Combination of melamine and cyanuric acid (each at dose: 25 mg/kg/day). \*\* indicated significant difference compared with the control group,  $p < 0.01$ .

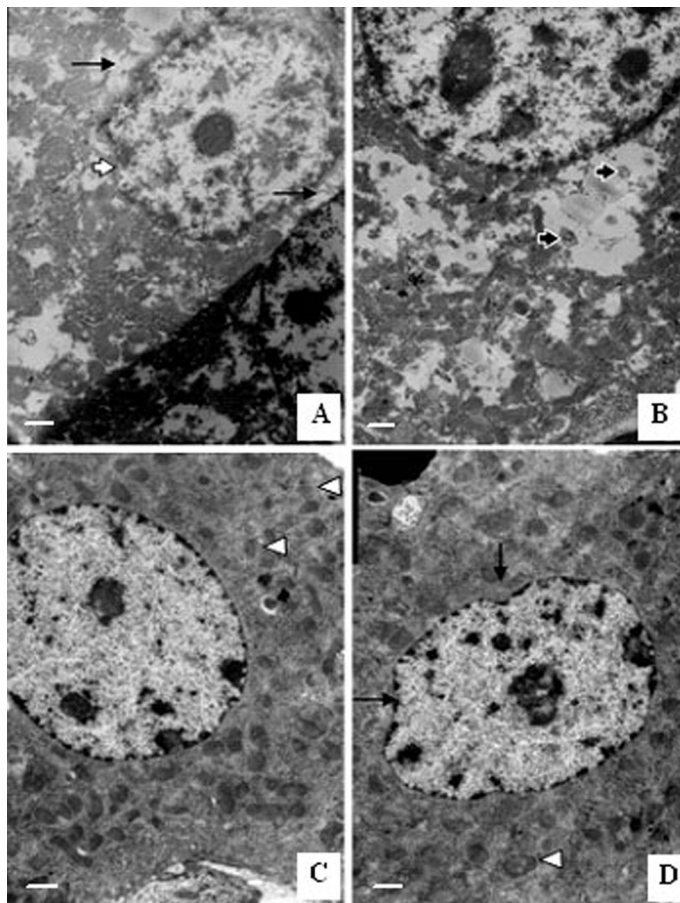
cant differences in body weight between control group and melamine group with a dose at 50 mg/kg/day (Figure 1).

### Pathological findings

At necropsy, the kidneys of surviving mice appeared swollen and pale yellow in color at the end of the experiment. No obvious changes were noted in the other organs.

### Ultrastructure changes of liver in mice

No obvious ultrastructure change was observed in hepatocytes of the animals from control group. However, as observed from Figure 2, the mice from melamine group exhibited ir-



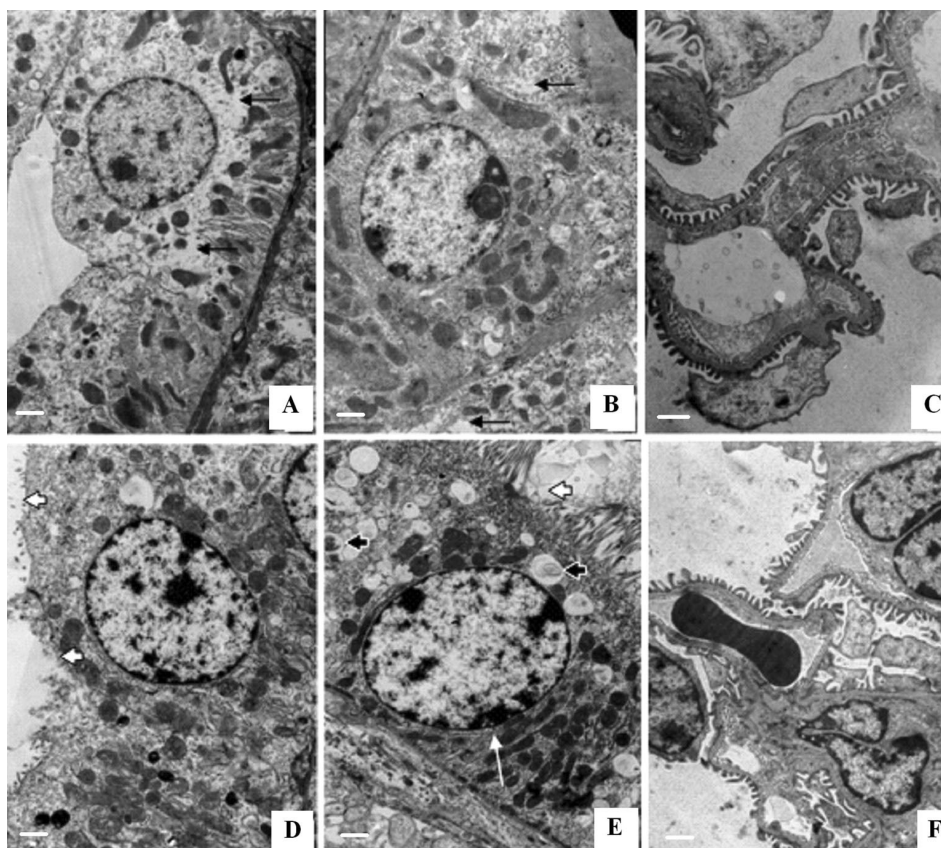
**Figure 2:** Ultrastructure changes in liver sections of mice. A and B represented the melamine group (50 mg/kg/day), as well as, C and D represented the co-administration group of melamine and cyanuric acid (each at: 25 mg/kg/day). The irregular nucleus shapes were indicated by the white short arrow. The lysis of some cytoplasm was indicated by white long arrow. The phagolysosomes were indicated by black short arrows. The sunken nuclei membrane was indicated black long arrows. The mitochondrials, which had indistinct outer membrane with a reduction of cristae, were indicated by white triangles. Scale bars = 1  $\mu$ m.

regular shape of nucleus, and indistinct nuclei membranes with the lysis of parts of the cytoplasm (Figure 2A). We observed more mitochondria, rough endoplasmic reticulum and lipid droplets in the cytoplasm. Also, phagolysosomes displaying medullary structure were noted in the cytoplasm of some cells (Figure 2B). The mice from co-administration group of melamine and cyanuric acid exhibited sunken nuclei membrane (Figure 2D), condensation of heterochromatin in nuclei with the presence of a large number of rough endoplasmic reticulum, mitochondria, ribosomes and lysosomes. Additionally, some mitochondria had indistinct outer membranes with a reduction of cristae (Figure 2C). These observations indicated that melamine (50 mg/kg/day) or its combination with cyanuric acid (each at 25 mg/kg/day) resulted in observable ultrastructural pathological changes in the liver of mice.

### Ultrastructure changes of kidney in mice

Based on electron microscopy examination, in comparison to the animals from control group, the animals from melamine group (50 mg/kg/day) exhibited less microvilli in the epithelial cell surface of distal tubule with the lysis of some cytoplasm, in which were observed large number of mitochondria, ribosomes, and pinocytotic vesicles (Figures 3A and B). Similar ultrastructural changes were also observed in the epithelial cell of proximal tubules, and dense microvilli were noted in the cell surface. No obvious pathological change was observed in glomeruli (Figure 3C). Co-administration of melamine and cyanuric acid (each at 25 mg/kg/day) resulted in more severe ultrastructural changes in the kidney of treated animals. There were short microvilli in the epithelial cell surface of distal tubule, even a local loss of microvilli was observed (Figure 3D). Margination of heterochromatin

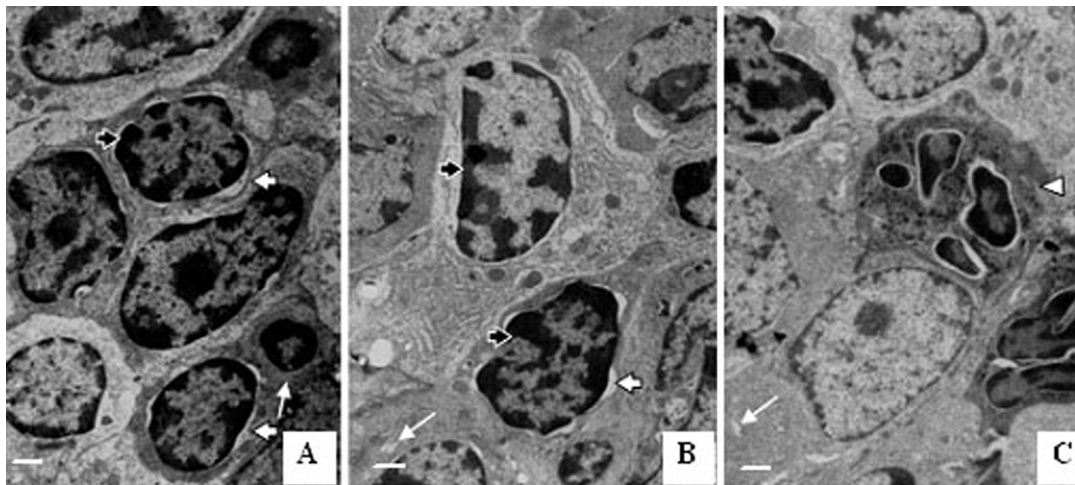
was present in nuclei, and phagolysosomes displaying medullary structure were also observed in cytoplasm (Figure 3E). There were large number of mitochondria, ribosomes, and pinocytotic vesicles in the cytoplasm of the proximal and distal tubular epithelial cells (Figure 3E). However, glomeruli exhibited basically normal structure with the presence of red blood cells in the capillary lumen (Figure 3F).



**Figure 3:** Ultrastructural changes of kidney tissue in mice. A, B and C represented melamine group (A: distal tubules, B: proximal tubules and C: glomerulus). D, E and F represented mixture group of melamine and cyanuric acid (D: distal tubules, E: proximal tubules and F: glomerulus). The local loss of microvilli was indicated by white short arrow. The margination of heterochromatin was indicated by white long arrow. The phagolysosomes were indicated by black short arrows. The lysis of some cytoplasm was indicated by black long arrow. Scale bars = 1  $\mu$ m.

### Ultrastructure changes of spleen in mice

The animals from control group presented a normal structural cytological structure pattern on electron microscopy examination. However, noticeable pathological injury in spleen tissue was observed in the animals from melamine group. The ingestion of melamine (50 mg/kg/day) resulted in marked edema



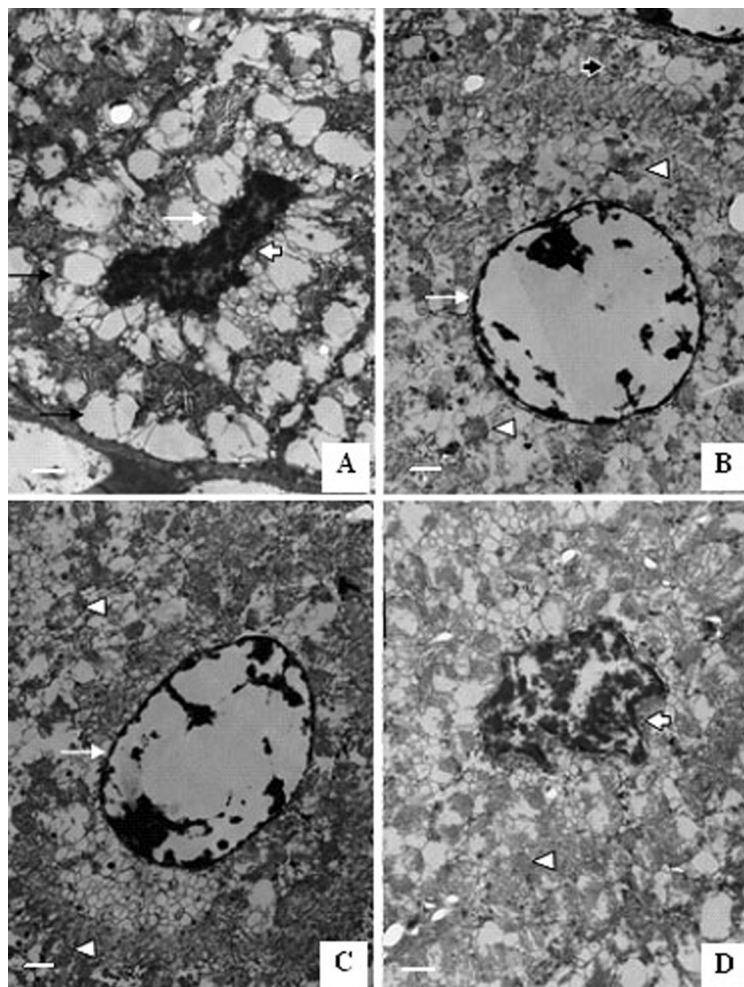
**Figure 4:** Ultrastructural changes of spleen tissue in mice. A represented melamine group, while B and C represented mixture group of melamine and cyanuric acid. The edema of nuclei membrane was indicated by white short arrow. The slight dilatation of rough endoplasmic reticulum was indicated by white long arrow. Black short arrow was used for indicating the obvious increasing in heterochromatin with the presence of heterochromatin margination. The white triangle was used for indicating the granulocytosis in blood sinus. Scale bars = 1 $\mu$ m.

of lymphocyte membrane (Figure 4A), indistinct membranes of some nuclei with irregular nuclear shapes. There was a clear increasing in heterochromatin with the presence of heterochromatin margination. Slight dilatation of rough endoplasmic reticulum also was observed in the cytoplasm of lymphocytes (Figure 4A). Moreover, splenic reticuloendothelial cells presented indistinct membranes with local edema. Co-administration of melamine and cyanuric

acid caused irregular nuclear shapes with local edema of nuclei membrane in lymphocytes and reticular epithelial cells (Figure 4B). Dilatation of rough endoplasmic reticulum was observed in reticular epithelial cells (Figure 4B and C). Additionally, the mice also displayed granulocytosis in the blood sinuses.

#### Ultrastructure changes of stomach wall in mice

In the mice from control group, no obvious ultrastructure change was observed in the cells of the stomach wall. However, the mice treated with melamine alone (50 mg/kg/day) presented with some irregular nuclear shapes with indistinct nuclei membranes (Figure 5A) or more space between cells (Figure 5B). There was a decrease of chromatin in nucleus, and condensation of heterochromatin in some cells (Figure 5A). There was large number



**Figure 5:** Ultrastructural changes of stomach wall in mouse. A and B represented melamine group, while C and D represented mixture group of melamine and cyanuric acid. White short arrow was used for indicating irregular nucleus shape and indistinct nuclei membrane. White long arrow was used for indicating the condensation of heterochromatin. Black short arrow was used for indicating the phagolysosome. Black long arrow was used for indicating the vacuolar degeneration of mitochondria. White triangle was used for indicating the damage of cristae and outer membrane of mitochondria. Scale bars = 1 $\mu$ m.

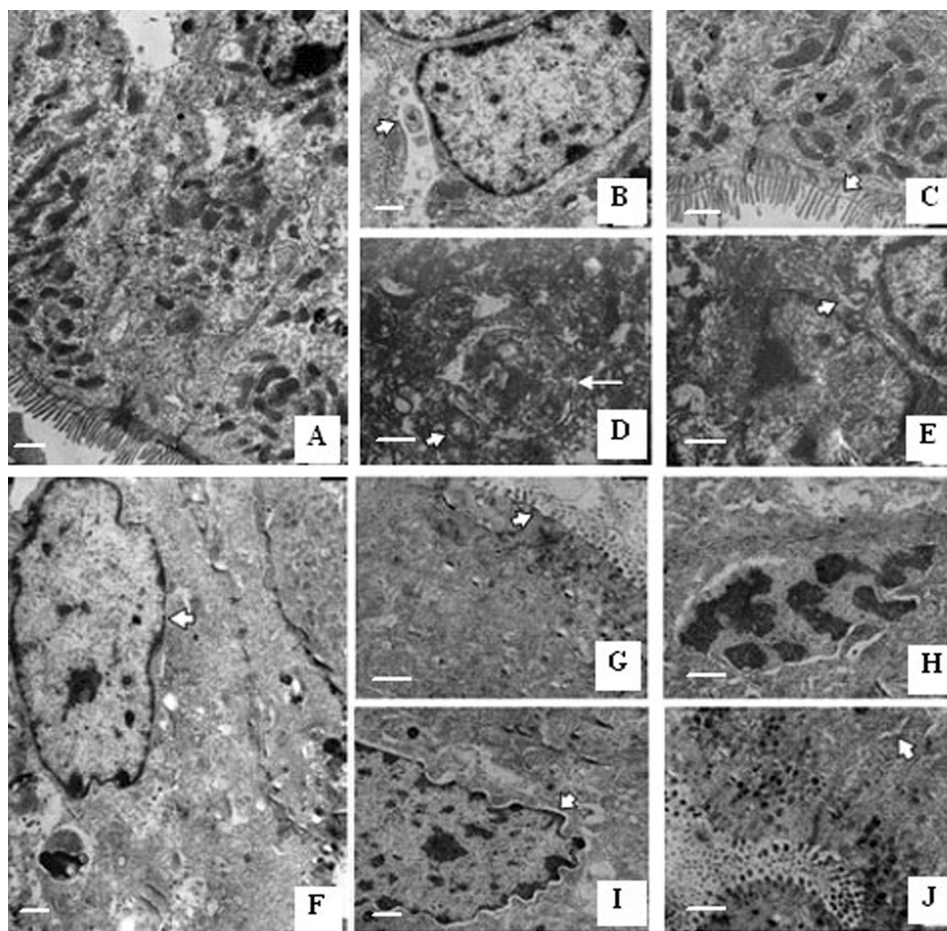
of smooth endoplasmic reticulum. We also observed vacuolar degeneration of mitochondria with the damage of cristae and outer membrane in some mitochondria (Figure 5B); phagolysosomes were visible (Figure 5B). Co-administration of melamine and cyanuric acid resulted in similar changes to that of melamine group, but the fracture and dissolution of some mitochondria cristae were observed in the animals from combination group of melamine and cyanuric acid (Figure 5C and D).

### Ultrastructure changes of small intestine in mice

No obvious ultrastructure change was observed in the cells of small intestine of the animals from control group. However,

the ingestion of melamine alone (50 mg/kg/day) resulted in local defect of microvilli in the surface of small intestine epithelial cells (Figure 6C); the cells exhibited irregular nuclear shape with the presence of heterochromatin margination. A large number of mitochondria, ribosomes, and lysosomes were present in the cytoplasm. Many mitochondria had indistinct outer membranes (Figure 6D). Phagolysosomes (Figure 6B) and membranous inclusions were visible, and some mitochondria presented with a reduction in their cristae (Figure 6D). The dilation of rough endoplasmic reticulum was also noted (Figure 6E). Co-administration of melamine and cyanuric acid caused more severe pathological changes in epithelial cell of small intestines, such as irregular nucle-

ar shapes, condensation and margination of heterochromatin (Figure 6F), thickening of nuclei membranes (Figure 6I). On the other hand, apoptotic cells were ruptured by nuclei fragmentation of equal size (Figure 6H). In cytoplasm, we also observed the dilation of rough endoplasmic reticulum (Figure 6J), and an increased number of lysosomes. In addition, small intestinal epithelial cells possessed short and sparse microvilli on their surface (Figure 6G).



**Figure 6:** Ultrastructure changes of small intestine in mice. A, B, C, D, and E represented melamine group, while F, G, H, I, and J represented mixture group of melamine and cyanuric acid. A: a large number of mitochondria, ribosomes, and lysosomal in cytoplasm; B: phagolysosome in cytoplasm. C: local defect of microvilli in the surface of small intestines epithelial cells. D: reduction of mitochondria cristae (indicated by short arrow) and membranous inclusions (indicated by long arrow). E: dilation of rough endoplasmic reticulum. F: irregular nucleus shape and margination of heterochromatin. G: short and sparse microvilli in the surface of small intestines epithelial cells. H: nuclei fragmentation from apoptosis cells. I: the thickening of nuclei membrane. J: The dilation of rough endoplasmic reticulum. Scale bars = 1  $\mu$ m.

### DISCUSSION

Melamine is primarily used in the manufacture of amino resins and plastics, and it is prohibited to exist in food however it may present only in small quantities (16). The WHO has set the tolerable daily intake of melamine at 0.2 mg/kg/day (17). Previously, melamine was also explored as a potential anti-cancer agent, but was afterwards discarded because of its insufficient benefits and its

potential toxicity (18). The most common toxic phenomenon is crystalluria, kidney stones and nephrotoxicity (1). Male mice have been shown to be more sensitive to melamine toxicity (19). Therefore, the male animals were used in the present study.

Histopathological methods are commonly used for detecting and evaluating organ-specific effects related to chemical exposure (20, 21). In the present work, based on the electron microscopic examination, the ingestion of melamine alone with a dose at 50 mg/kg/day resulted in observable ultrastructural cellular pathological injury to kidney, liver, spleen, stomach wall and small intestinal wall of male mice. Compared with that of melamine alone (50 mg/kg/day), co-administration of melamine and cyanuric acid (each at dose: 25 mg/kg/day) caused more severe ultrastructural cellular pathological changes to the examined visceral organs, including kidney, liver, spleen, stomach wall and small intestines wall. The results support the assumption that the toxicity of melamine may not be limited to renal stone formation as in animal studies where melamine was present in high dosages or in combination with cyanuric acid (1). On the other hand, our results from the present work were also consistent with the viewpoint that toxic effects of melamine were further aggravated by the presence of cyanuric acid (1).

Park *et al.* (2011) showed that the co-administration of melamine and cyanuric acid (each at 50 mg/kg), resulted in numerous crystals present in the distal tubules and collecting ducts of the rats, moreover, the high dose of melamine and cyanuric acid (each at 400 mg/kg) caused the severe dilation of renal tubules, and glomerular atrophy, as well as numerous hyaline droplets which accumulated in the epithelial cells of proximal tubules (22). In the present work, co-administration of melamine and cyanuric acid (each at 25 mg/kg/day) resulted in the shortening of microvilli in the epithelial cell surface of distal tubules, and even a local loss of microvilli (Figure 3D). On the other hand, we also observed margination of heterochromatin in nucleus, and phagolysosomes in the cytoplasm with the presence of a large number of mitochondria, ribosomes, and pinocytotic vesicles in the cytoplasm of proximal and distal tubular epithelial cells. In the animals from melamine group (50 mg/kg/day), less microvilli also was observed in the epithelial cell surface of distal tubules with the lysis of some cytoplasm. In our work, however, no toxic electron microscopic lesions were observed in glomeruli of the animals treated

with 25 mg/kg of melamine plus cyanuric acid or 50 mg/kg of melamine alone.

The nucleus is one of the most prominent cellular organelles within a eukaryotic cell, and altered nuclei shape is considered to be important for cell function (23). In the present work, the ingestion of melamine alone or combination of melamine and cyanuric acid caused the presence of irregular nuclear shapes in the liver, spleen, stomach wall, and small intestinal wall. Although, it is still not entirely clear how nuclear shape affects function, it has been speculated that changes in nuclei shape might lead to changes in chromosome organization, which in turn can affect gene expression (24). On the other hand, an abnormal nuclei shape is also associated with cancer (25), although the functional relationship between altered nuclei shape and cellular transformation is not clear. This was consistent with the viewpoint that exposure on the melamine may be carcinogenic (19).

The condensation of nucleus is believed to be one of the two major morphological features of apoptosis, the cell suicide program (9). In the present study, the administration of melamine alone or the combination of melamine and cyanuric acid resulted in the condensation of nucleus, and margination of chromatin were observed in hepatocytes, stomach wall and small intestinal wall of treated mice, implying that apoptosis was occurring in the cells. Similar apoptosis were also reported by Xie *et al.* in the liver of mice (10) and in differentiated PC12 cell by Han *et al.* (11). The genes *Bax* and *Caspase-3* were shown by Xie *et al.* to be involved in the melamine-induced apoptosis (10). Mitochondrial function is important in the regulation of cellular life and death, including disease states, and the disturbance in mitochondrial function and distribution can be accompanied by significant morphological alterations (26). In the present work, we also observed vacuolar degeneration of mitochondria with the damage of cristae and outer membrane in some mitochondria (Figure 5B). On the other hand, a large number of mitochondria were present in cytoplasm of cells of the liver, kidney and small intestine cells. These ultrastructure changes may indicate a phenotypic general mitochondrial dysfunction.

Recently, Pirarat *et al.* (2012) investigated the pathological effects of feeding melamine and cyanuric acid, separately or in combination, to walking catfish (*Clarius batrachus*) (27). They showed that melamine-related crystals were distributed multifocally throughout the liver, kidney, heart, and spleen of

fish fed melamine and cyanuric acid in combination. Also, Reimschuessel *et al.* (2008) reported the presence of crystals in the intestinal tract of fish exposed to melamine and cyanuric acid (6).

A dearth of information is available on the melamine-related toxic effects on spleen, stomach wall, and small intestine wall in mammals. In this study, to the best of our knowledge, for the first time we have demonstrated that pathological effects are present in the spleen, stomach wall, and small intestine of mice treated with both melamine alone and combination of melamine and cyanuric acid. Moreover, in comparison to the melamine treated group (50 mg/kg/day), more severe apoptosis was observed in co-administration groups of melamine and cyanuric acid (each at 25 mg/kg/day). However, melamine-related crystal formation was not observed in the treated mice, which may be related to the strain of mice used in this study. However, further studies would be necessary to reach a final conclusion.

To the best knowledge of the authors, this is the first report of melamine-related ultrastructural cell damage in organs other than the kidney and liver in mammals. This finding suggests that melamine alone or its combination with cyanuric acid appears to be systemically toxic to mice. Recent studies demonstrated that melamine could effectively inhibit outward potassium currents and delay rectified potassium currents in rat hippocampal CA1 neurons (28). Also, studies have shown that melamine not only inhibits differentiated PC12 cell proliferation, but also affects the morphology and caspase-3 activity of hippocampal neurons even at low doses (9, 10). Together with our results, it was suggested that the toxicity of melamine appears to have been underestimated. Therefore, a larger investigation of melamine toxicity on different tissue and organs should be conducted for a more complete toxic profile of melamine.

In conclusion, following the exposure to melamine alone or its combination with cyanuric acid for 14 consecutive days, our results demonstrated that melamine alone (50 mg/kg/day) resulted in certain ultrastructural pathological injury to liver, kidney, spleen, stomach wall, and small intestine of mice. The combination of melamine and cyanuric acid (each at 25 mg/kg/day) was clearly shown to be more toxic to the examined organs than melamine alone. These results would be a supplement to the existing toxic profile of melamine. Also, they may be helpful in understanding melamine-induced pathology of in mammals.

## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

## ACKNOWLEDGEMENTS

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