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The toxic effect of a mixture of melamine and cyanuric acid on the gastrointestinal tract and liver in mice

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ABSTRACT

The aim of this study was to investigate the effects of a mixture of melamine (MA) and cyanuric acid (CA) on the gastrointestinal tract and liver in mice. Kunming mice were given 0, 10, 100, or 200 mg/kg.bw/day MA and CA mixture (MC, each compound) in corn oil by gavage for 7 consecutive days. Autopsy showed severe renal injury in all MC-treated mice and histopathological examination revealed dose-related lesions in the gastrointestinal tract and liver other than the kidneys. Subsequently, Kunming mice were given 0, 0.3, 1.5, or 7.5 mg/kg·bw/day MC (each compound) in corn oil by gavage for 28 consecutive days. The results showed that higher doses of MC caused mortality and alteration on the body weights, relative liver weights, and blood chemistry parameters related to treatment. Histopathologically, the liver revealed scattered hepatic necrosis and apoptosis. Villous height and villus-to-crypt depth ratios were decreased in the duodenum and jejunum, with marked expression of proliferating cell nuclear antigen in the epithelium compared with controls. In conclusion, MC mixture could cause toxic effects in the gastrointestinal tract and liver in mice during acute and sub-acute toxicity study.

Studies on the combined toxicity of melamine (MA) and cyanuric acid (CA) have become a new topic in veterinary medicine. For example, the interaction between MA and CA was responsible for the 2007 outbreaks of acute renal failure in pets in North America (Brown et al., 2007; Dobson et al., 2008), because the two compounds easily form an insoluble precipitate in kidney tubules which cause renal failure by some combination of epithelial necrosis and physical blockage (Kobayashi et al., 2010). Subsequently, the renal toxicity of a mixture of MA and CA (MC) was demonstrated extensively in many experimental animals (Puschner et al., 2007; Reimschuessel et al., 2008; Xie et al., 2009). Until now, limited information is available regarding the effects of MC on organs other than kidneys. Therefore, the purpose of this study was to evaluate the effects of MC on the gastrointestinal tract and liver in mice.

Initially, a 7-day trial study was designed to determine the possible adverse effects of MC on other organs as well as kidneys. Therefore, 32 male Kunming mice were randomly divided into four groups ($n = 6$ per group). MC (MA + CA, 1:1 w/w) or vehicle alone (control) was given to mice by oral gavage at doses of 0, 10, 100, 200 mg/kg·bw/d (each compound) suspended in corn oil. All mixtures were given daily for 7 days, and the mice were euthanized on the 8th day. The selection of the highest dose was based on the study of Dobson (Dobson et al., 2008).The organs were removed and fixed in 2.5% paraformaldehyde–glutaraldehyde solution for histopathology examination. The fixed organs were embedded in paraffin wax, sectioned at 4 μm, and stained with hematoxylin and eosin. The melamine–cyanurate crystals were detected by Oil Red O staining (Kobayashi et al., 2010). The Institutional Animal Care and Committee of China Agricultural University approved all the experimental procedures.

In the 7-day study, all the animals treated with MC at all doses died or needed to be euthanized before day 8 because of severe renal toxicity.

Histopathologically, the typical lesions of crystal deposition and renal tubular necrosis were observed in the MC-treated kidneys. In addition, obvious damages were seen in the tissues of the gastrointestinal tract, liver (Fig. 1 A1, B1, and C1) and testes.

Interestingly, we also found melamine–cyanurate crystals in the gastrointestinal tract and liver in the highest dose group (Fig. 1 A2, B2, and C2). The crystals exhibited red in color by Oil Red O staining (Fig. 1 A3, B3, and C3). This findings indicated that high dose of MC could form crystals in other organs rather than kidneys. It is commonly believed that exposure to MC does not cause crystal formation in any other part of the body other than the kidney. However, one report indicated that such crystals were distributed multifocally throughout the liver, kidney, heart, and spleen in fish fed MC (Pirarat et al., 2012). Our findings provide further evidence regarding the distribution of melamine–cyanurate crystals in the body following the administration

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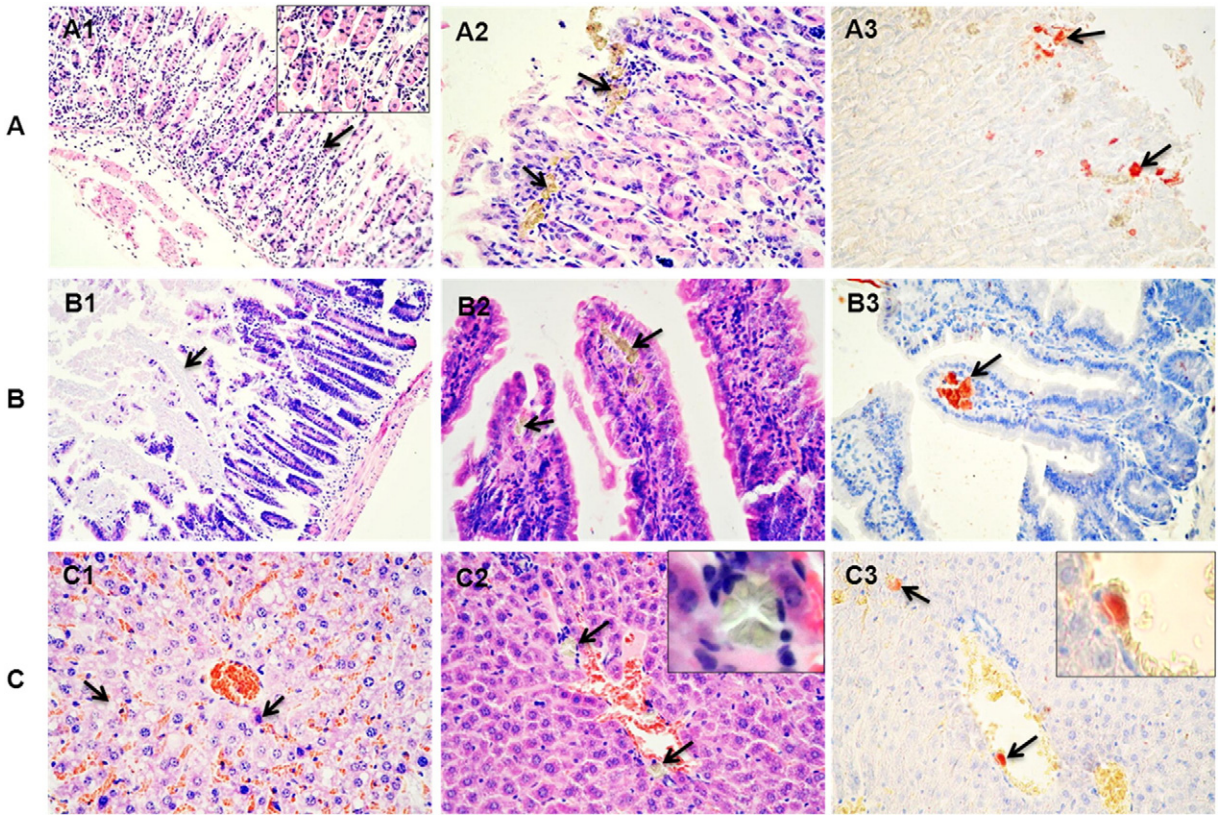

Fig. 1. Histopathology findings in the gastrointestinal tract and liver taken from mice treated with high doses of MC for 7 days. A. In the stomach, the mucosa showed diffuse necrosis and lymphocytic infiltrations (A1, arrow). Several yellow crystals were found in the top of the mucosa (A2, arrow), and they stained red with Oil Red O (A3, arrow, B3 and C3). B. In the small intestine, the mucosa was markedly necrotic (B1, arrow). Crystal deposition was found in the villous layer (B2 and B3, arrows). C. In the liver, hepatocytes showed fatty changes and scattered necrosis (C1, arrow). Crystals were occasionally seen in the liver tissue, and the crystals located in the sinuses (C2, arrows) or in the portal vein (C3, arrows).

of MC, and this result suggested that MC might have a direct irritation on the gastrointestinal tract and liver.

Due the 7-day study revealed that MC could cause toxic effects on the gastrointestinal tract, liver and testes other than the kidney, a 28-day sub-acute study was carried on to further characterize the effects of MC on these organs. Our lab had demonstrated the sub-acute toxicity of MC on the testes in mice (You et al., 2012; Chang et al., 2014). In this study, we focused on describing the effect of MC on the gastrointestinal tract and liver in the same animal model.

For the 28-day study, 48 Kunming mice were randomly divided into four groups ($n = 12$ each group). MC was given to mice by oral gavage at dose levels of 0, 0.3, 1.5 or 7.5 mg/kg·bw/d (each compound) as a suspension in corn oil. The dose selection was based on the 7-day study. All mixtures were given daily for 28 days, and the mice were euthanatized on the 29th day.

The serum levels of blood urea nitrogen (BUN), creatinine (Cr), aspartate transaminase(AST), and alanine transaminase(ALT) were assayed by Sino-UK Institute of Biological Technology, Beijing, China. The organs were removed and fixed in 2.5% paraformaldehyde–glutaraldehyde solution for histopathology. An identical small intestinal segment was analyzed in each case using an Image Analysis System (Olympus 6.0, Olympus, Tokyo, Japan). The villus height and crypt depth of at least 5 well-oriented villi were measured in each specimen (Xu et al., 2003; Wu et al., 2004). The expression levels of cell proliferation marker proliferating cell nuclear antigen (PCNA) were measured immunohistochemically to determine the numbers of proliferating cells in the intestinal epithelium (Raab et al., 1998). Levels of PCNA in the sections were quantified using a Motic Med 6.0 CMIAS Image Analysis System (Motic China Group Co., Ltd., Beijing, China). A total of 30 fields per mouse (10 fields per section and three sections per mouse) were chosen randomly and analyzed. The positive staining intensity was calculated as the ratio between the stained area and the total analyzed field at a magnification 400. Apoptosis in the liver was detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. The sections of liver tissue were prepared and stained with an in situ cell death detection kit following the manufacturer's protocol (Roche, Basel, Switzerland). TUNEL-positive cells were evaluated using a light microscope under high power magnification ($\times 400$) in a blinded fashion. For each tissue section, five fields were selected randomly and the total number of TUNEL-positive cells was counted. The results are expressed as the mean $\pm$ standard deviation. Differences between groups were determined with a one-way analysis of variance followed by a least significant difference post-hoc test using SPSS version 16.0 software (IBM Corp., Armonk, NY, USA).

In the 28-day study, one mouse in the 1.5 mg/kg/d MC group and three mice in the 7.5 mg/kg/d MC group died during the experimental period. In the 7.5 mg/kg/d group, the body weights and relative liver weights decreased significantly compared with the controls (data not shown). Serum chemistry data showed that AST and ALT levels increased markedly in the 7.5 mg/kg/d MC group compared with the controls (data not shown), indicating that MC damaged liver function. Histopathologically, scattered or small foci of necrosis of hepatocytes were seen in the MC-treated mice, and the degree of the damage was in a dose-dependent manner (Fig. 2A). In the 7.5 mg/kg/d MC group, marked apoptosis was observed in the liver tissues by TUNEL staining ($P < 0.01$, Fig. 2B, G). Regarding the gastrointestinal tract, no or only slight lesions were seen in all treatment groups. However, decreases in duodenal and jejunal villous height and villus-to-crypt depth ratios