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Evaluation of Renal Toxicity by Combination Exposure to Melamine and Cyanuric Acid in Male Sprague-Dawley Rats

Ji Yeon Son[1], Yoon Jong Kang[1], Kyeong Seok Kim[1], Tae Hyung Kim[1], Sung Kwang Lim[1], Hyun Jung Lim[2], Tae Cheon Jeong[3], Dal Woong Choi[4], Kyu Hyuck Chung[1], Byung Mu Lee[1] and Hyung Sik Kim[1]

[1]*Division of Toxicology, School of Pharmacy, Sungkyunkwan University, Suwon, Korea*
[2]*College of Pharmacy, Pusan National University, Busan, Korea*
[3]*College of Pharmacy, Youngnam University, Gyeongsan, Korea*
[4]*Department of Public Health Sciences, Graduate School, Korea University, Seoul, Korea*

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Melamine-induced nephrotoxicity is closely associated with crystal formation in the kidney caused by combined exposure to melamine (Mel) and cyanuric acid (CA). However, there are few dosage-finding studies for toxicological evaluation of chronic co-exposure to Mel and CA. The objective of this study was to investigate the possible mechanism by which a Mel and CA mixture lead to renal toxicity in rats. Mel and CA were co-administered to rats via oral gavage for 50 days. Nephrotoxicity was determined by measuring blood urea nitrogen (BUN) and serum creatinine (sCr) levels. Relative kidney weights were significantly increased in rats after co-exposure to Mel+CA (63/6.3 or 630/6.3 mg/kg) mixtures. BUN and sCr levels were significantly increased after Mel and CA co-exposure. Taken together, significant increase in KIM-1, NGAL, and calbindin levels were observed in the urine of rats exposed to Mel+CA (63/6.3 or 630/6.3 mg/kg) compared with the corresponding control group. Histological analysis revealed epithelial degeneration and necrotic cell death in the proximal tubules of the kidney after co-exposure to Mel+CA (63/6.3 or 630/6.3 mg/kg). Our data suggest that Mel-mediated renal toxicity may be influenced by CA concentrations in Mel-contaminated milk or foods.

***Key words*:** Melamine, Cyanuric acid, Nephrotoxicity, Mixture, Chronic toxicity

INTRODUCTION

In 2008, Chinese infant formula contaminated with 1,3,5-triazine-2,4,6-triamine (melamine, Mel) was found to induce severe nephrotoxicity in babies (1). Most of these children were younger than 3 years old, but many older children were reported to have acute kidney toxicity. In particular, Mel and stones were detected in the urine or kidneys of the affected infants (2,3). Based on these reports, the Korea Food and Drug Administration (KFDA) immediately inspected all products imported from China that may have been contaminated with Mel. Although a large number of products contained low levels of Mel, some crackers were highly contaminated, with Mel levels of approximately 300 mg/kg (4). Similarly, Mel-contaminated food or food ingredients imported from China were recalled from the United States, Canada, Singapore, and Hong Kong (5,6).

Mel was found in foods such as milk and powdered infant formula, because of the reckless actions of manufacturers seeking to increase the protein content of their products. Several Mel-related compounds, such as ammeline (4,6-diamino-5*H*-1,3,5-triazine-2-one), ammelide (6-amino-1*H*-1,3,5-triazine-2,4-dione), and cyanuric acid (1,3,5-triazine-2,4,6-triol, CA), are byproducts of the manufacture of Mel. In a previous study by The U.S. National Toxicology Program (7), Mel did not induce renal toxicity after subchronically or chronically exposed in mice and rats. CA does not induce severe toxicity in rats, mice, and dogs based on acute, subchronic, or chronic toxicity studies (8,9). The acute oral LD_{50} of Mel and CA is > 3 g/kg body weight. In addition, the no observed adverse effect level (NOAEL) of Mel was found to be 63 mg/kg/day based on kidney toxic-

Correspondence to: Hyung Sik Kim, Laboratory of Molecular Toxicology, School of Pharmacy, Sungkyunkwan University, Chunchun-dong 300, Changan-gu, Suwon, Kyunggi-do 440-746, Korea
E-mail: hkims@skku.edu

ity in a 13-week toxicity study (10,11). Although Mel and CA individually have low acute toxicities, severe kidney toxicity in animals is observed when animals have combined exposure to Mel and CA (12,13). Mel-induced renal dysfunction is suspected to involve crystal formation by Mel and CA binding in the kidney, but the exact mechanism of action is unknown (14). Previous studies have shown that Mel–CA crystals have a rounded shape *in* vivo (15,16) but are needle-like *in vitro*, and that the crystal shape is unaffected by pH (17). Recently, there have been investigations into the mechanism by which the combination of Mel+CA inducing renal toxicity and kidney stone formation (9,15,16).

For this research, we conducted a comparative evaluation of nephrotoxicity after exposure to Mel, CA, or a mixture of Mel and CA in Sprague-Dawley rats. Although Mel and CA mixtures can cause renal toxicity, the exact mixture ratios that induce renal toxicity are not known. We investigated Mel-cyanurate renal stone formation and determined the mixture ratios at which Mel+CA-induced renal toxicity in rats. We also conducted morphological observations of Mel-cyanurate crystals in kidney tissue and compared these to crystals formed by Mel+CA mixtures *in vitro*.

MATERIALS AND METHODS

Animal and chemical treatments. Sprague-Dawley male rats (3 weeks-old, 130~150 g) were obtained from Charles River Laboratories (Japan) and housed in an environment with controlled temperature (22 ± 2°C) and lighting (12 hr light/dark cycles). The animals were given food (PMI, Brentwood, MO, USA) and tap water *ad libitum*. All animals were randomly assigned into five groups (6 animals per group) and acclimatized for one week prior to the experiment. The dosages (63 mg/kg/day) of Mel or CA used in this study were based on the no observed adverse effect level (NOAEL) from a 13-week toxicity assay (U.S. National Toxicology Program, 1983). The CA in the mixture was initially believed to be present at an approximately tenfold lower concentration than Mel, hence the 63/6.3 dose ratio.

Mel (63 mg/kg), CA (63 mg/kg), and a mixture of Mel+CA (63/6.3 or 630/6.3 mg/kg) were administered orally for 50 days. The vehicle control group was administered 1% CMC-Na in the same manner. The volume of each dose was adjusted to 5 ml/kg based on the daily body weights obtained just before dosing. The body weight of each animal was recorded daily. After the final treatment, both kidneys (right and left) and the urinary bladder were excised and weighed. The liver, testes, epididymis, thyroid glands, and adrenal glands were also weighed to investigate whether any of these organs were secondary targets of Mel or the mixture of Mel+CA. The left kidney was immediately frozen in liquid nitrogen and stored at −80°C. The right kidney was fixed overnight in 10% neutral formalin and dehydrated in 70% ethanol. The institutional animal care committee of Pusan National University approved the experimental protocol (approval number: PNU-2012074). The experimental procedures were carried out in accordance with the international guidelines for care and use of laboratory animals.

Clinical signs and body weight changes. Throughout the study period, each animal was observed at least once daily for clinical signs of toxicity related to the chemical treatment. On working days, all cages were checked in the morning and afternoon for dead or moribund animals.

Urinalysis and serum biochemical parameters. After the final chemical treatment, urine was collected over a 24 hr period using metabolic cages. The rat urine samples were collected and immediately centrifuged at 13,000 rpm (15,700 *g*) for 10 min. Urinalysis was performed on fresh urine samples from individual animals, and various parameters, including total protein, creatinine, and glucose levels, were determined using a urine chemistry analyzer (TBA-200FR NEO, Toshiba, Japan). Total urine volume and urinary pH were also recorded. Urinary sediments, including red blood cells and crystals, were counted under an optical microscope (Olympus Corporation, Tokyo, Japan).

Rats were fasted overnight and anesthetized with CO_2 gas. Blood was collected from the abdominal aorta and immediately centrifuged at 3000 *g* for 10 min. The sera were immediately stored at −80°C prior to the analysis of alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, blood urea nitrogen (BUN), and uric acid using a Hitachi 912 automatic analyzer (Roche Diagnostics, Manheim, Germany). Creatinine clearance was calculated using urine volume, plasma and urine creatinine levels in the collected 24 hr urine sample.

Histological examination. For the hematoxylin and eosin (H&E) staining, several consecutive 5-μm paraffin sections were de-paraffinized with xylene for 7 min (3 times). After gradual rehydration in a series of graded alcohols and washing with deionized water, the sections were stained with H&E for 1 min, rinsed with deionized water, and developed in tap water for 5 min. The tissue sections were destained by dipping the slide in acidified ethanol and rinsing in tap water. After washing with deionized water, the sections were stained with eosin for 30 sec, dehydrated, and mounted. The histopathological findings were determined using a photonic microscope.

Immunohistochemical analysis. A 5-μm-thick section of paraffin-embedded tissue was de-paraffinized and rehydrated through a series of grade alcohols. Endogenous peroxidase activity was blocked by 30 min incubation in a 3% hydrogen-peroxide-methanol solution followed by a wash

in phosphate-buffered saline (PBS). The slides were boiled in 0.01 M citrate buffer (pH 6.0) in a microwave oven for 18 min to enhance the immunoreactivity of the anti-proliferative cellular nuclear antigen (PCNA) antibodies (Upstate Biotechnology, Lake Placid, NY, USA). After washing, the slides were incubated with the anti-PCNA antibody (dilution, 1 : 200) for 2 hr at 4°C and incubated for 30 min with biotinylated goat anti-rabbit IgG immunoglobulin (Zymed Laboratories, San Francisco, CA, USA). Next, the slides were again rinsed in PBS, followed by incubation with a horseradish-peroxidase (HRP)-streptavidin-conjugated secondary antibody (Zymed Laboratories; dilution, 1 : 200) for 2 hr, and labeled with an aminoethyl carbazole substrate kit (Zymed Laboratories) for 5 min or until an adequate signal was observed. Slides were then washed in distilled water, mounted with a glycerol vinyl alcohol mounting solution (Zymed Laboratories), and observed under light microscopy.

Protein extraction and Western blot analysis. Preserved urine samples were mixed with an activation/wash buffer and the resulting slurry with bound proteins was loaded onto a mini filter spin column. The bound urine proteins were washed to remove any remaining impurities. Finally, the purified total urine proteins were eluted into 150~300 µl of the provided elution buffer. The method employed removes highly concentrated salts and metabolic wastes to allow for isolation of high quality proteins. The protein concentration in the tissue extract was determined using a protein assay reagent (Bio-Rad, Hercules, CA, USA). Extracted proteins were denatured by boiling in a sample buffer (0.5 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.1% bromophenol blue, 10% β-mercaptoethanol) at 96°C for 5 min. A total of 50 µg protein was separated via 8~15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for 90 min at 100 V using a running buffer, and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) for 90 min at 100 V using transfer buffer. The membranes were blocked for 1 hr in TNT buffer (10 mM Tris-Cl, pH 7.6, 100 mM NaCl, and 0.5% Tween 20) containing 5% skim milk and incubated overnight at 4°C with specific primary antibodies. After washing for 1 hr with TNT buffer, the membranes were incubated with HRP-conjugated anti-rabbit, anti-mouse or anti-goat antibodies (1 : 10,000) for 1 hr at room temperature and subsequently washed for 1 hr with TNT buffer. Proteins were detected using ECL plus Western blotting reagents (Amersham Biosciences, Buckinghamshire, UK). An image analyzer was used to determine the relative band intensities.

Detection of newly developed urinary biomarkers. The evaluation of novel urinary kidney markers was performed using WideScreen™ Rat Kidney Toxicity Panel 1. This panel is based on an immunosandwich assay immobilized on microparticle beads and was used according to the manufacturer's instructions, as described by Hoffmann *et al*. (18).

In vitro Mel and CA crystallization. Pure-grade samples of Mel and CA (1 : 1) were taken and a hot solution of CA was added dropwise to a hot solution of Mel. The mixed Mel and CA solution was stirred for nearly 4 hr and then allowed to cool at room temperature. A white-colored Mel–CA crystal was obtained, washed with distilled water to remove impurities, and dried at a constant temperature of 80°C. The crystal compound was analyzed by scanning electron microscopy (SEM).

Statistical analysis. All values are expressed as mean ± S.E.M. Statistical analysis was performed with *SigmaStat* Ver. 3.5 (SPSS Inc., Chicago, IL, USA). The significance of differences between the control and treatment groups was determined using a one-way analysis of variance (ANOVA), followed by Bartlett's test. A p value < 0.05 was considered to be statistically significant.

RESULTS

Clinical signs, body, and organ weight changes. No clinical signs of toxicity or deaths occurred in any of the treatment groups during the experimental period. The body weight of rats significantly increased in the group treated with Mel (63 mg/kg) after a 25 day exposure (Fig. 1). However, a significant reduction in body weight was observed in groups treated with the Mel+CA mixture (630/6.3 mg/kg). The absolute and relative thyroid gland weights were significantly increased in the Mel+CA mixture groups compared

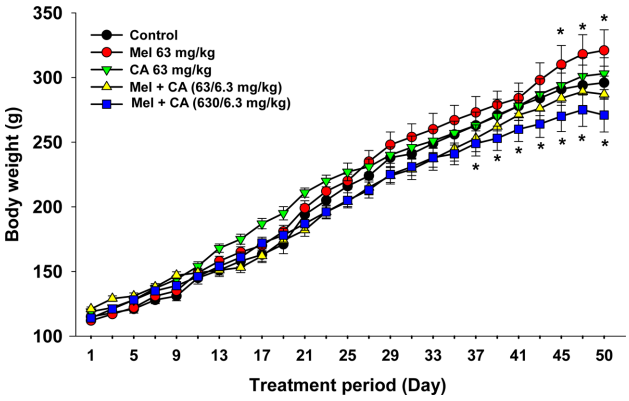

Fig. 1. Change in the body weights of Sprague-Dawley rats treated with melamine, cyanuric acid and a mixture of melamine/cyanuric acid. Melamine (Mel, 63 mg/kg), cyanuric acid (CA 63 mg/kg) or Mel/CA (63/6.3 mg/kg and 630/6.3 mg/kg) was administered daily to Sprague-Dawley rats via oral gavage for 50 days. The data are expressed as mean ± S.E.M. of 6 rats/group. The asterisk indicates a significant difference from the control ($p < 0.05$).

Table 1. Organ weight changes in Sprague-Dawley rats after 50 days of exposure to melamine, cyanuric acid, or a mixture of melamine and cyanuric acid

Groups	Control	Mel (63 mg/kg)	CA (63 mg/kg)	Mel+CA (63/6.3 mg/kg)	Mel+CA (630/6.3 mg/kg)
Absolute weight					
Liver (g)	9.97 ± 1.15[a]	10.51 ± 0.67	9.44 ± 0.97	9.21 ± 0.20	9.22 ± 0.29
Kidney (g)	1.10 ± 0.14	1.26 ± 0.04	1.21 ± 0.13	1.14 ± 0.09	1.12 ± 0.06
Testis (g)	1.29 ± 0.05	1.45 ± 0.05	1.34 ± 0.10	1.48 ± 0.05	1.29 ± 0.06
Epididymis (g)	0.65 ± 0.05	0.67 ± 0.03	0.62 ± 0.01	0.56 ± 0.04*	0.55 ± 0.02*
Thyroid glands (mg)	8.52 ± 2.14	9.12 ± 1.58*	9.03 ± 2.10*	11.09 ± 3.72*	13.38 ± 2.21*
Adrenal glands (mg)	53.40 ± 5.41	52.38 ± 4.98	53.72 ± 5.63	51.80 ± 5.06	46.08 ± 4.06
Relative weight					
Liver (mg/g b.w.)	33.54 ± 0.85	32.68 ± 1.40	31.06 ± 2.73	32.08 ± 1.13	34.13 ± 2.65
Kidney (mg/g b.w.)	3.70 ± 0.29	3.93 ± 0.14	3.98 ± 0.34	3.98 ± 0.25	4.14 ± 0.25*
Testis (mg/g b.w)	4.83 ± 0.32	4.52 ± 0.16	4.41 ± 0.20	5.14 ± 0.14	4.77 ± 0.39
Epididymis (mg/g b.w.)	2.22 ± 0.39	2.07 ± 0.12	2.06 ± 0.19	1.95 ± 0.14*	1.96 ± 0.31*
Thyroid glands (mg/g b.w.)	0.03 ± 0.01	0.05 ± 0.01*	0.05 ± 0.01*	0.05 ± 0.02*	0.04 ± 0.02*
Adrenal glands (mg/g b.w.)	0.18 ± 0.02	0.16 ± 0.01	0.18 ± 0.01	0.18 ± 0.02	0.17 ± 0.02

Abbreviations: Mel, melamine; CA, cyanuric acid.
[a]Data are expressed as mean ± SEM (n = 6).
*Significant difference from the control group at $p < 0.05$.

with the control group, but epididymis weights were markedly decreased in the Mel+CA mixture groups (Table 1). The relative kidney weights were significantly increased in the group treated with the Mel+CA (630/6.3 mg/kg) mixture compared with the control group, whereas the Mel (63 mg/kg) and CA (63 mg/kg) treatments did not significantly change the kidney weights (Table 1). These results indicate that the mixture of Mel+CA had a negative impact on kidney weight.

Serum biochemistry and urinalysis. Serum biochemistry results are shown in Table 2. The mean BUN and sCr levels were significantly increased in the Mel+CA mixture groups compared with the control group, suggesting renal toxicity due to the Mel+CA mixture. In addition, serum uric acid levels were significantly higher in the Mel+CA mixture groups compared with the control group, but the AST and ALT levels did not change among the treatment groups. Na, K, and Ca levels were significantly increased in the Mel+CA mixture groups compared with the control group, but blood phosphorus levels decreased (Table 2). The urine volume was significantly increased in the Mel+CA (63/6.3 and 630/6.3 mg/kg) groups compared with the control, whereas the urinary pH was significantly reduced in the Mel+CA mixture groups. Interestingly, round-type crystals and blood were found in the urine collected from the Mel+CA mixture groups, indicating that the exposure to the Mel+CA mixture resulted in urinary excretion of Mel+CA

Table 2. Serum biochemical parameters of Sprague-Dawley rats after 50 days of exposure to melamine, cyanuric acid, or a mixture of melamine and cyanuric acid

Groups	Control	Mel (63 mg/kg)	CA (63 mg/kg)	Mel+CA (63/6.3 mg/kg)	Mel+CA (630/6.3 mg/kg)
ALT (U/L)	56.52 ± 2.93[a]	52.92 ± 3.65	50.38 ± 6.81	62.34 ± 8.75	62.32 ± 8.74
AST (U/L)	141.32 ± 15.76	138.52 ± 15.6	131.71 ± 18.97	124.54 ± 18.61	124.52 ± 18.62
BUN (mg/dl)	15.42 ± 2.84	16.39 ± 5.72	19.63 ± 4.35	32.51 ± 5.42*	32.57 ± 5.43*
Creatinine (mg/dl)	0.56 ± 0.07	0.54 ± 0.03	0.59 ± 0.09	1.57 ± 0.45*	1.57 ± 0.45*
Uric acid (mg/dl)	1.63 ± 0.21	1.19 ± 0.05	1.52 ± 0.28	2.12 ± 0.43*	2.27 ± 0.84*
Phosphorus (mg/dl)	7.56 ± 1.24	5.43 ± 0.82	6.72 ± 1.34	3.63 ± 1.28*	2.3 ± 1.5*
Na (mg/dl)	125.4 ± 24.3	132.6 ± 12.8	126.9 ± 5.7	137.8 ± 10.2*	139.5 ± 8.12*
K (mg/dl)	5.61 ± 0.83	5.27 ± 0.85	5.94 ± 1.82	6.24 ± 1.91*	5.23 ± 1.14
Ca (mg/dl)	8.57 ± 2.11	8.45 ± 2.34	8.72 ± 1.97	9.21 ± 2.35*	9.82 ± 1.73*

Abbreviations: Mel, melamine; CA, cyanuric acid; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen.
[a]Data are expressed as mean ± SEM (n = 6).
*Significant difference from the control group at $p < 0.05$.

Table 3. Urine biochemistry alterations of Sprague-Dawley rats after 50 days of exposure to melamine, cyanuric acid, or a mixture of melamine and cyanuric acid

Parameters \ Groups	Control	Mel (63 mg/kg)	CA (63 mg/kg)	Mel/CA (63/6.3 mg/kg)	Mel+CA (630/6.3 mg/kg)
Urine volume (ml/day)	10.4 ± 0.9[a]	7.5 ± 1.2	12.5 ± 0.8	13.8 ± 1.8*	22.4 ± 1.9*
Urinary pH	7.7 ± 0.1	7.1 ± 0.4	7.0 ± 0.4	6.9 ± 0.4*	6.7 ± 0.4*
Hemorrhage	-	-	-	+	+
Crystal	-	-	-	+	+
Specific gravity	1.015	1.254	1.031	1.358*	1.427*
Protein (mg/dl)	140.5 ± 41.3	250.4 ± 25.9	158.3 ± 21.7	107.2 ± 34.6*	98.3 ± 25.4*

Abbreviations: Mel, melamine; CA, cyanuric acid.
[a]Data are expressed as mean ± SEM (n = 6).
*Significant difference from the control group at $p < 0.05$.

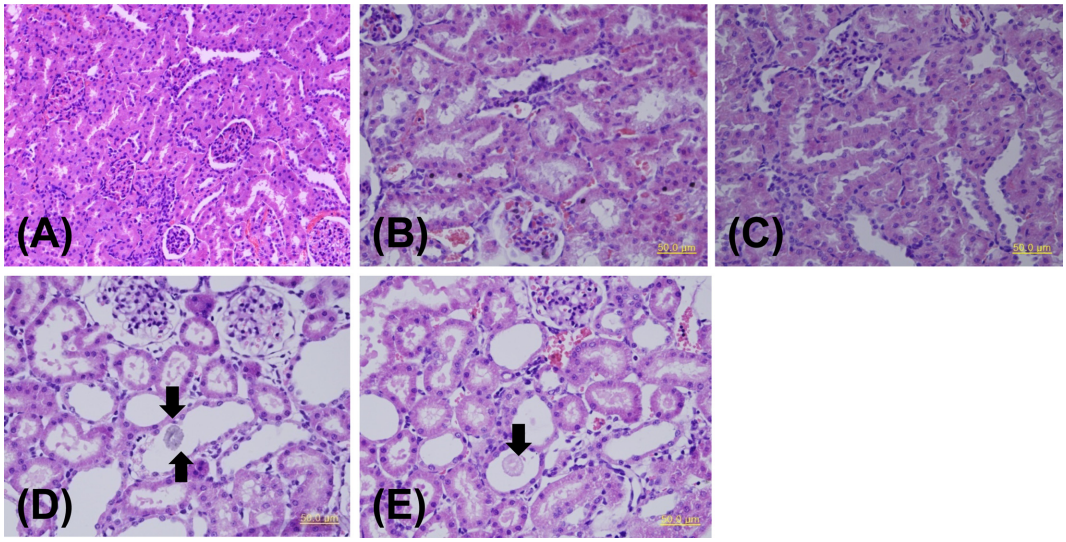

Fig. 2. Changes in histological examination of the kidney after 50 days of exposure to melamine, cyanuric acid or a mixture of melamine/cyanuric acid. Histological findings in H&E stained kidney tissues from the (A) control group, (B) Mel group, (C) CA group, and (D and E) Mel+CA (63/6.3 and 630/6.3 mg/kg) mixture groups. Magnification is 100×. Arrows indicate the renal crystals found in the renal cortex of a whole kidney tissue section (bar scale = 15 μm).

crystals formed in the rat kidney (Table 3).

Histological examination and immunohistochemistry. In accordance with the serum biochemistry data, edematous and yellowish kidneys were observed in the group treated with Mel+CA (63/6.3 mg/kg) compared with those of the control group (data not presented). Microscopically, damaged tubular epithelial cells were markedly induced in the Mel+CA mixture groups. In particular, variable grades of inflammation were noted in the Mel+CA mixture groups. Moreover, crystalline deposits were found throughout the distal and proximal tubules (Fig. 2D), which indicate that Mel+CA crystals formed along the osmotic gradient of the kidney. Only the animals with crystals in their kidneys showed increased BUN and creatinine, indicating that the renal crystals indeed impaired renal function. To identify proliferating cells in rat kidneys, we probed kidney tissue sections with the proliferation marker PCNA. In the Mel+CA mixture groups, we observed cells that were strongly PCNA-positive in the kidney (Fig. 3).

Changes in urinary protein biomarkers. Previous studies have used the excretion levels of KIM-1, NGAL, and calbindin proteins as biomarkers of acute kidney injury (19,20). To determine whether the urinary excretion of these proteins correlated with Mel-induced kidney injury, an enzyme immunoassay was performed to the urine of rats treated with Mel, CA, or a mixture of Mel+CA. Significant increase in KIM-1, NGAL, and calbindin levels were observed in the urine of rats exposed to Mel+CA (63/6.3 or 630/6.3 mg/kg) compared with the corresponding control group (Fig. 4). Similar to BUN and sCr levels, there were no significant differences in these acute kidney injury biomarkers in groups treated with Mel (63 mg/kg) or CA (63

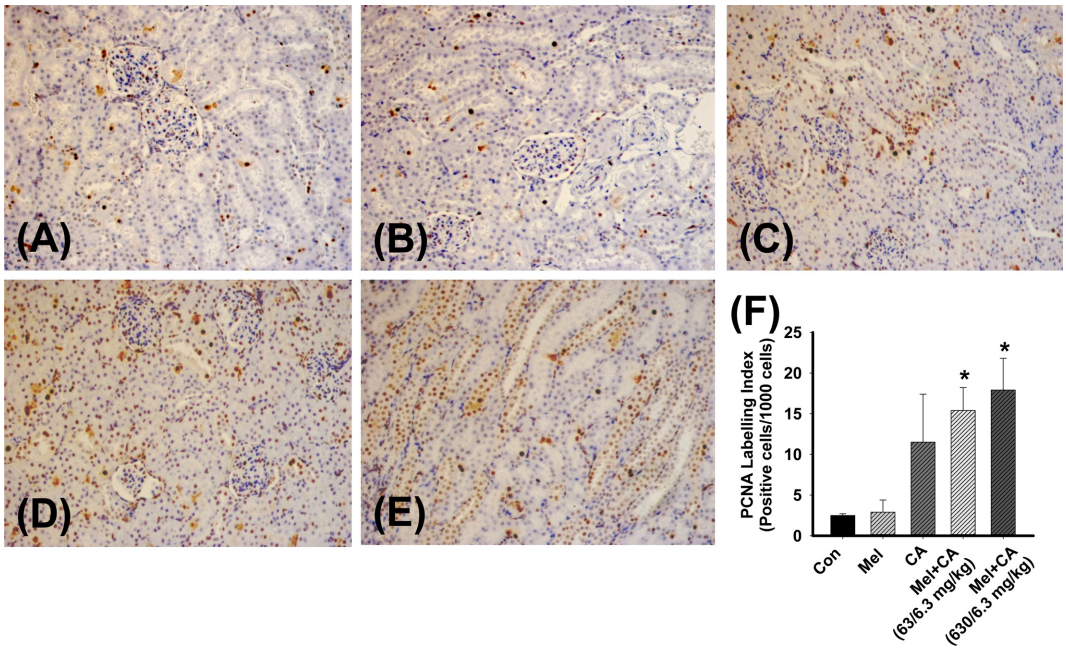

Fig. 3. Immunohistochemical expression of proliferative cellular nuclear antigen (PCNA) in the kidneys of rats treated with melamine, cyanuric acid or a mixture of melamine/cyanuric acid for 50 days. Immunohistochemical localization of PCNA in the (A) control group, (B) Mel 63 mg/kg group, (C) CA 63 mg/kg group, (D) Mel+CA mixture (63/6.3 mg/kg) group, and (E) Mel+CA mixture (630/6.3 mg/kg) group. Magnification is 400×. These results are representative of 6 animals. (F) Expression of PCNA-positive cells in each group was counted under a light microscope for 40 fields in the cortex under 200× magnification. The asterisk indicates a significant difference from the control (*$p < 0.05$).

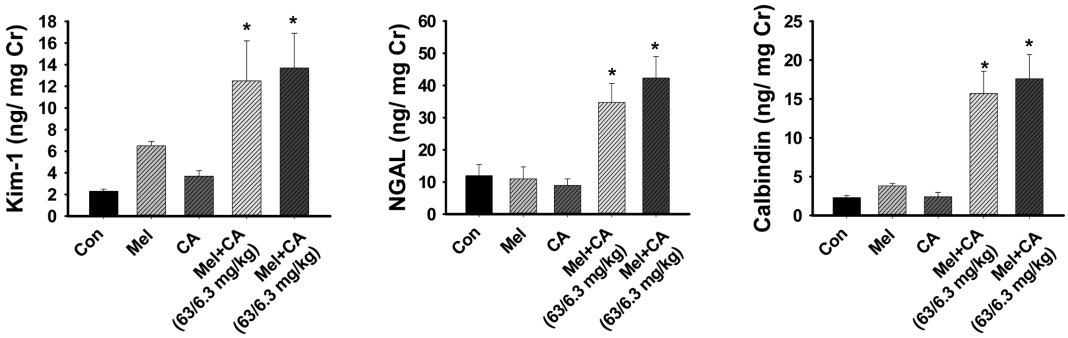

Fig. 4. Changes of urinary biomarkers in Sprague-Dawley rats following exposure to melamine, cyanuric acid, or a mixture of melamine/cyanuric acid. Male rats were administered saline (control), Mel (63 mg/kg), CA (63 mg/kg), or Mel+CA mixtures (63/6.3 or 630/6.3 mg/kg) for 50 days. Urine was collected and urinary excretion levels of biomarkers (Kim-1, NGAL, and calbindin) were assayed using WideScreen™ Rat Kidney Toxicity Panel 1. The statistical significance of differences between control and treated groups was determined using Bonferroni's multiple comparison tests. The asterisk indicates a significant difference from the control ($p < 0.05$).

mg/kg) alone (Fig. 4).

SEM analysis of crystal morphology. SEM was used to study surface features in Mel–CA crystals. Fig. 5 shows different types of morphologies exhibited by Mel, CA, and Mel–CA crystals at different magnifications. The shape of Mel–CA crystals formed *in vivo* was rounded, as shown in Fig. 2. However, we observed needle-like crystal formation *in vitro*. Fig. 5 shows an SEM photograph of crystals formed by a Mel–CA mixture.

DISCUSSION

A previous study demonstrated that co-exposure to Mel/CA (1 : 1 ratio) caused acute kidney injury in experimental animals (15). Taken together, recent data have also demonstrated that the kidney is the primary target organ for Mel-mediated toxicity, but also that Mel alone does not induce

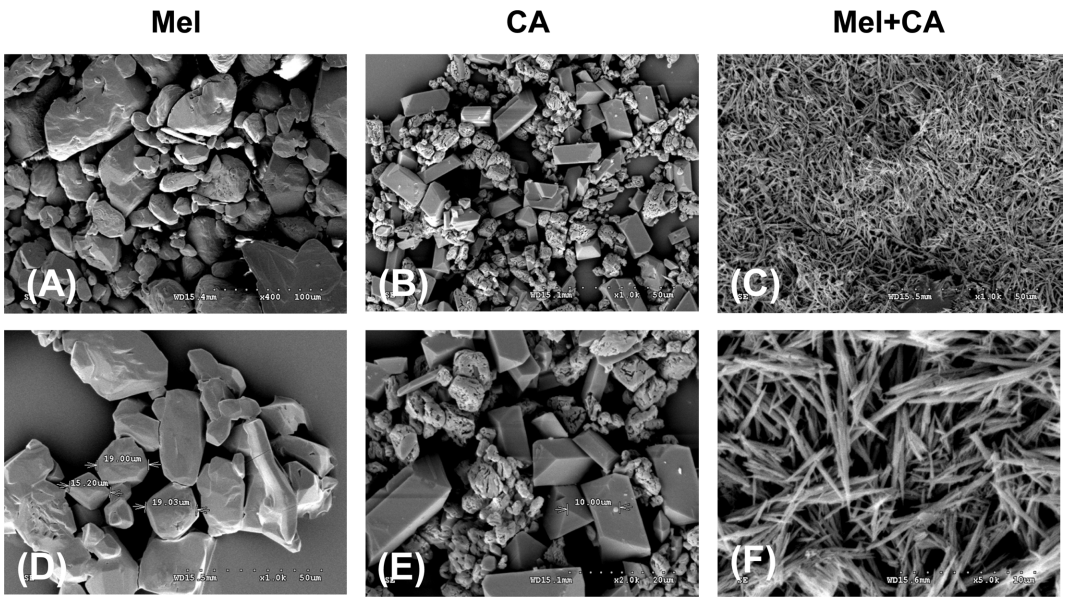

Fig. 5. Morphology of melamine, cyanuric acid, and melamine-cyanurate crystals *in vitro* as observed by scanning electronic microscope. Magnifications are ×100 (A,B,C) and ×50 (D,E,F). Mel–CA crystals had needle-like shapes (C and F).

severe nephrotoxicity in experimental animals (21,22). However, many recent cases of acute renal injury are closely related to the ingestion of Mel-contaminated powdered infant formula imported from China. Recently, Mel was found to contain several metabolites, such as ammeline, ammelide, and CA, and Mel was used for the adulteration of foods or milk to increase their apparent protein content. Mel and its metabolites are assumed to be absorbed by the gastrointestinal tract and then precipitate in the kidney to form crystals (22,23). Previous studies have demonstrated that oral administration of a mixture of Mel+CA leads to crystal formation in the kidney or urinary tract and that these crystals can cause tubular obstruction in the kidney, ultimately leading to renal injury and acute renal failure (24-26). Although Mel mixed with CA has been shown to induce toxic effects, little is known about the co-exposure ratio of Mel+CA mixture and how it relates to Mel-induced renal injury.

In the present study, we observed crystal formation in the renal tubules of rats after chronic co-exposure to a Mel+CA (63/6.3 mg/kg) mixture. To clearly investigate the mechanism underlying Mel–CA crystal formation, it is necessary to study the ratio of Mel to CA that results in formation of crystals. We demonstrated that BUN and sCr levels were significantly elevated in Sprague-Dawley rats treated with Mel+CA (63/6.3 or 630/6.3 mg/kg) mixtures. Histological examination revealed renal crystal formation in affected kidney tissue only in groups treated with Mel+CA mixtures, and these crystals were detected mainly in the cortex. However, groups treated with Mel (63 mg/kg) or CA (63 mg/kg) alone did not show any kidney damage. Therefore, we suggest that co-exposure to both Mel and CA induces substantial renal toxicity in animals. These results were similar to those of a previous study in which Mel+CA (12/12 mg/kg/day) was co-administered to rats (27).

The pathogenesis of Mel/CA-induced renal toxicity might be renal filtration injury caused by Mel–CA crystals. Urine examination showed that urine volume significantly increased in the groups receiving Mel+CA mixtures. Urinary pH levels were significantly decreased, and protein concentration was also decreased in groups treated with Mel+CA mixtures. It was thus of interest to determine which components of the total protein were affected. Among the urinary protein components, protein biomarkers of kidney injury induced by Mel+CA, including KIM-1, NGAL, and calbindin proteins, were significantly increased. These results indicate that urinary calbindin, NGAL, and KIM-1 may be sensitive indicators of nephrotoxicity. These biomarkers were previously characterized as early and sensitive protein biomarkers in urine, as evidenced by significant increases in the cortex of renal injury (28,29).

To confirm the crystal formation caused by Mel+CA mixtures, SEM was used to study the surface features of the Mel–CA crystal. As shown in Fig. 5, Mel–CA crystals were seen with polarized light optical microphotography. The Mel–CA crystals formed *in vivo* were rounded, but needle-like crystal formation was observed *in vitro*. A previous study had suggested that pH is a factor that might affect Mel–CA crystal formation and morphology (26). We confirmed that, *in vitro*, needle-like Mel–CA crystals formed in suspensions with neutral pH, suggesting that pH is not involved in the formation of rounded Mel–CA crystals.

This study clearly demonstrated that rounded Mel–CA crystals in the body might be associated with serum proteins, minerals, and urine pH.

The results of the present study provide new insights into mechanism of toxicities induced by mixtures of Mel+CA. The WHO (5) has established a tolerable daily intake (TDI) of Mel at 0.2 mg/kg body weight, which is applicable to humans of all ages but only applicable to exposure to Mel. If humans are exposed to mixtures of Mel+CA, crystal formation in the kidney may be influenced by the low concentrations of CA.

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