Dose-Response Assessment of Nephrotoxicity from a 7-Day Combined Exposure to Melamine and Cyanuric Acid in F344 Rats

Cristina C. Jacob,* Renate Reimschuessel,† Linda S. Von Tungeln,* Greg R. Olson,‡ Alan R. Warbritton,‡ David G. Hattan,§ Frederick A. Beland,* and Gonçalo Gamboa da Costa*¹

*Division of Biochemical Toxicology, National Center for Toxicological Research, Jefferson, Arkansas 72079; †Center for Veterinary Medicine, Laurel, Maryland 20708; ‡Toxicologic Pathology Associates, Jefferson, Arkansas 72079; and §Center for Food Safety and Applied Nutrition, College Park, Maryland 20740

¹To whom correspondence should be addressed at Division of Biochemical Toxicology, National Center for Toxicological Research, HFT-233, 3900 NCTR Road, Jefferson, AR 72079. Fax: (870) 543-7136. E-mail: goncalo.gamboa@fda.hhs.gov.

Received August 6, 2010; accepted October 24, 2010

The intentional adulteration of pet food with melamine and derivatives, including cyanuric acid, has been implicated in the kidney failure and death of a large number of cats and dogs in the United States. Although individually these compounds present low toxicity, coexposure can lead to the formation of melamine cyanurate crystals in the nephrons and eventual kidney failure. To determine the dose-response for nephrotoxicity upon coadministration of melamine and cyanuric acid, groups of male and female F344 rats (six animals per sex per group) were fed 0 (control), 7, 23, 69, 229, or 694 ppm of both melamine and cyanuric acid; 1388 ppm melamine; or 1388 ppm cyanuric acid in the diet for 7 days. No toxicity was observed in the rats exposed to the individual compounds, whereas anorexia and a statistically significant increase in blood urea nitrogen and serum creatinine levels was observed in the animals treated with 229 and 694 ppm melamine and cyanuric acid. The kidneys of these animals were grossly enlarged and pale yellow. Large numbers of crystalline structures deposited in the tubules were seen on sections in kidneys from all rats in these treatment groups. No significant changes were detected in the remaining treatment groups exposed to both melamine and cyanuric acid. In the melamine-only treatment group, 5 of 12 rats had scattered crystals present in renal tubules when examined by wet mount. These were not observed by histopathology. The observed adverse effect level (8.6 mg/kg bw [body weight]/day) and benchmark dose modeling data (8.4–10.9 mg/kg bw/day) determined in this study suggest that the tolerable daily intake values derived from studies conducted with melamine alone may underestimate the risk from coexposures to melamine and cyanuric acid.

Key Words: melamine; cyanuric acid; melamine cyanurate; nephrotoxicity.

Melamine (1,3,5-triazine-2,4,6-triamine, CAS 108-78-1) is a high production volume chemical that is used extensively by industry in the preparation of polymers for manufacturing of a broad range of products, including laminates, adhesives,

houseware items, and flame retardants (Crews *et al.*, 2006). Cyanuric acid (1,3,5-triazine-2,4,6-triol, CAS 108-80-5), a deaminated derivative of melamine, is also a high production volume chemical used to prepare herbicides, dyes, resins, and antimicrobial agents and as a stabilizer and disinfectant in swimming pool water (Huthmacher and Most, 2003).

Upon the sudden illness and death of a large number of cats and dogs in the spring of 2007 and the subsequent voluntary mass recall of pet food by a major manufacturer, the U.S. Food and Drug Administration (USFDA) initiated a wide-scale investigation into the cause of these illnesses and deaths. The intentional adulteration of wheat and rice gluten, which was used in the preparation of pet food, with “scrap melamine” was soon identified as the probable cause for the observed toxicities. Scrap melamine is a residue from the industrial synthesis of melamine that contains varying proportions of a number of oxytriazines, including cyanuric acid. Given the high nitrogen content of melamine and related triazines, the adulteration was conducted to increase artificially the nitrogen content of gluten and, thus, its estimated protein content and commercial value. Further confirmation that the deaths were due to exposure to melamine and cyanuric acid was obtained upon the necropsy of a number of affected animals: melamine cyanurate (CAS 37640-57-6) crystals in the kidneys were identified as the probable cause for the observed nephrotoxicity and eventual renal failure (University of Guelph, 2007).

The bulk of data available in the literature indicates that neither melamine nor cyanuric acid alone poses a significant acute toxicological hazard to a number of mammalian species, with the LD₅₀ values for the individual compounds approaching the LD₅₀ for NaCl (LD₅₀ rat, oral = 3161 mg/kg for melamine; LD₅₀ mouse, oral = 3296 mg/kg for melamine; LD₅₀ rabbit, dermal > 1000 mg/kg for melamine; LD₅₀ rat, oral > 10,000 mg/kg for cyanuric acid; LD₅₀ rabbit, dermal > 7940 mg/kg for cyanuric acid) (Hammond *et al.*, 1986; NTP, 1983; OECD, 1998, 1999).

Although a wealth of data is available regarding the toxicity of exposure to melamine or cyanuric acid individually in a number of species (WHO, 2009), the available toxicological data for combined exposures to melamine and cyanuric acid are still limited with regard to the range of doses, number and sex of test animals, and route of administration. Reports of experiments conducted in various animal species, including fish (Reimschuessel *et al.*, 2008), hogs (Reimschuessel *et al.*, 2008), cats (Puschner *et al.*, 2007), and rats (Chen *et al.*, 2009; Dobson *et al.*, 2008; Kim *et al.*, 2010; Kobayashi *et al.*, 2010; Xie *et al.*, 2010), indicate the presence of acute renal toxicity, with formation of tubule-obstructing crystalline spherulites in all these models, suggesting a common mode of toxicity. It has been proposed that when animals are exposed to mixtures of melamine and cyanuric acid, the compounds are absorbed in the gastrointestinal tract, distributed systemically, and precipitate in the kidney to form nephrotoxic melamine cyanurate crystals (Dobson *et al.*, 2008; Puschner *et al.*, 2007).

In order to obtain dose-response data for the induction of nephrotoxicity upon coexposure to melamine and cyanuric acid, we exposed groups of male and female F344 rats to 0 (control), 7, 23, 69, 229, or 694 ppm each of melamine and cyanuric acid; 1388 ppm of melamine alone; or 1388 ppm of cyanuric acid alone.

MATERIALS AND METHODS

Chemicals. Melamine (Sigma-Aldrich, St Louis, MO; stated purity 99%, CAS 108-78-1) and cyanuric acid (Fluka, anhydrous, stated purity > 98%) were obtained from Sigma-Aldrich. The purity and identity of the test articles were confirmed by high-pressure liquid chromatography (HPLC) coupled with ultraviolet detection and electrospray mass spectrometry and by gas chromatography-mass spectrometry in electron impact mode. All solvents and reagents used for the dosed-feed analyses were purchased from Sigma-Aldrich and met or exceeded American Chemical Society specifications.

Preparation of the dosed feed. The melamine and cyanuric acid were individually pulverized to fine powders with a manual mortar and pestle and incorporated in NIH-41 irradiated meal using a 2.5-cubic feet twin shell blender (Patterson-Kelley Co., East Stroudsburg, PA) to afford the concentrations of 0 (control), 7, 23, 69, 229, or 694 ppm each of melamine and cyanuric acid; 1388 ppm of melamine; or 1388 ppm of cyanuric acid. These concentrations were selected taking into consideration the average National Center for Toxicological Research (NCTR) breeding colony historical body weight and feed consumption of 6-week-old male and female F344 rats and aimed to afford exposures of, respectively, 0, 1, 3.3, 10, 33, and 100 mg/kg bw (body weight)/day each of melamine and cyanuric acid, 200 mg/kg bw/day melamine, or 200 mg/kg bw/day cyanuric acid. The control feed underwent the same mechanical blending procedure as the dosed-feed preparations.

Dosed-feed certification. Five-gram samples of each dosed- or control feed blend (nine samples per blend: three from the top, three from the middle, and three from the bottom) were analyzed for melamine and cyanuric acid by isotopic dilution tandem mass spectrometry (MS/MS). Briefly, the samples were spiked with appropriate amounts of $^{13}\text{C}_3$ -labeled cyanuric acid and $^{15}\text{N}_3$ -labeled melamine (Toronto Research Chemicals Inc., North York, Ontario, Canada) and extracted for 30 min in a sonicator bath with 25 ml of 2.5% HCl. The extracts were centrifuged to remove any insoluble matter, and the supernatants were diluted 1:100 in 99:1 acetonitrile/water and filtered with 0.2 μm polytetrafluoroethylene Whatman Mini-

UniPrep Syringeless Filters (Whatman Inc., Piscataway, NJ). The samples thus obtained were analyzed by HPLC-MS/MS using an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA) coupled to a Quantum Ultra MS/MS (Thermo Fisher Scientific Inc., Waltham, MA) operated in electrospray mode. The samples (1 μl) were eluted isocratically at 450 $\mu\text{l}/\text{min}$ with 10mM ammonium acetate in 92.8% acetonitrile in water using a ZIC-HILIC column (150 $\times$ 2.1 mm, 5 μm , SeQuant, Umeå, Sweden) thermostated at 35°C. The mass spectral analyses were conducted by multiple reaction monitoring in the positive (melamine) or negative (for cyanuric acid) electrospray mode. The monitored transitions for an argon collision cell pressure of 1.3 mTorr were as follows: m/z 128.0 $\rightarrow$ 42.0 at 16 eV (cyanuric acid), m/z 131.0 $\rightarrow$ 43.1 at 16 eV ($^{13}\text{C}_3$ -labeled cyanuric acid), m/z 127.1 $\rightarrow$ 85.1 at 12 eV (melamine), and m/z 130.1 $\rightarrow$ 87.1 at 11 eV ($^{15}\text{N}_3$ -labeled melamine). The quantification was based upon the relative integration of the unlabeled and labeled triazines. Plots of response ratios for labeled versus unlabeled melamine or cyanuric acid were linear ($r^2 = 0.9999$) over the concentration range of 10–3000 ppb unlabeled plus 25 ppb of labeled standards and had, respectively, slopes of 0.064 and 0.082. The method had an intra- and interday accuracy of 93.1–99.9% and a precision of 1.5–7.7% Relative Standard Deviation and indicated that the melamine and cyanuric acid were, respectively, at 109.3 ± 8.8 and $107.1 \pm 15.2\%$ of the target concentrations.

Animals and exposure. All procedures involving care and handling of animals were reviewed and approved by the NCTR Institutional Animal Care and Use Committee. F344 rats (six males and six females per dose group, 6 weeks old) were obtained from the breeding colony at the NCTR, weight ranked (acceptable weight: $\pm 20\%$ of the mean body weight), and randomly assigned to treatment groups that were fed *ad libitum* for 7 days with NIH-41 irradiated meal (control animals), NIH-41 irradiated meal containing melamine and cyanuric acid (combined exposure groups), or NIH-41 irradiated meal containing melamine or cyanuric acid (single-agent exposure groups). The animals were housed in individual polycarbonate cages with hardwood chip bedding, and the room environmental controls were set to maintain a relative humidity of 40–70% and a 12-h light/day cycle. Water and food consumption and body weights were measured daily.

Histopathological procedures. At the end of the 7-day exposure period, the animals were anesthetized by carbon dioxide inhalation and blood was withdrawn by cardiac puncture until exsanguination. A complete necropsy and detailed histopathological analysis of the kidneys were performed on all animals. At necropsy, the kidneys were weighed and sectioned longitudinally. One half of each kidney (right and left) was flash frozen for later wet-mount preparation. The other half of the left kidney was placed in 10% neutral buffered formalin (NBF) for 2 h, whereas the other half of the right kidney was sectioned in half once again, with one section (1/4 kidney) used immediately for wet-mount preparation and one section (1/4 kidney) placed in 10% NBF for 2 h.

The wet-mount analysis was conducted by pressing approximately 2-mm-thick sections of fresh tissue between two microscope slides and observation under bright field and polarized light microscopy. Presence of crystals was scored on a subjective scale from 0 to 5, with 0 = no crystals seen, 1 = one crystal in entire section, 2 = a few crystals with a scattered distribution, 3 = a moderate number of crystals seen throughout the section, 4 = large numbers, seen immediately when viewing under the microscope, and 5 = extensive numbers of crystals, obliterating the regular architecture of kidney.

The NBF-fixed tissues were trimmed, processed, embedded in Formula R (Surgipath Medical Ind., IL), sectioned at approximately 5 μm , and stained with hematoxylin and eosin (H&E).

In order to determine the occurrence of melamine cyanurate crystals in the urine, the urinary bladder was injected with a small volume of saline during the necropsy procedures, and the lavage specimen thus obtained was examined by bright field and polarized light microscopy.

Clinical chemistry. Terminal blood samples obtained by cardiac puncture were allowed to clot and then centrifuged. The serum was removed and frozen at -80°C until clinical chemistry analysis. Blood urea nitrogen (BUN) and creatinine levels were analyzed on an Alfa Wassermann ALERA analyzer

(West Caldwell, NJ). The instrument was calibrated daily, and two levels of assayed controls were included in daily analyses as internal controls.

Statistics. Bw data were analyzed with a repeated measures design in SAS using the general linear model, with day as the repeated factor. Pairwise comparisons to the control group were conducted with Dunnett's test.

Kidney weights, BUN, and creatinine levels were analyzed by one-way ANOVA, with pairwise comparisons to the control group conducted by Dunnett's test. When necessary, the data were transformed by taking the natural logarithm to obtain a more normal data distribution or equal variance.

RESULTS

Animal Morbidity and Mortality

One male and one female animals from the highest combined melamine and cyanuric acid dose treatment (694 ppm) died unexpectedly overnight after 2 days of exposure. The remaining animals from this treatment group were removed from the study the following morning, and samples were taken for clinical chemistry, wet-mount kidney sections, and histopathological analysis.

Effective Exposure

The effective mean daily dose of melamine and/or cyanuric acid in each group was determined taking into consideration the concentration of the triazines in the feed of each group and the average daily feed consumption and body weights of the animals. Given the similarity of the exposures obtained, the data were pooled for males and females within each dose group. Table 1 itemizes the effective exposures to melamine and cyanuric acid in each group in relation to the targeted dose for each group. The effective exposures ranged from 30 to 90% of the targeted doses, and a general trend was observed for a decrease in the effective doses in the combined treatment groups receiving the higher concentrations of melamine and cyanuric acid in the formulated feed.

Body Weights

The body weights of the animals were measured in the morning of each day of treatment (Supplementary fig. 1S).

TABLE 1

Target Doses and Average Effective Experimental Daily Doses of Melamine and/or Cyanuric Acid for Male and Female F344 Rats during the 7 Days of Treatment

Concentration in diet (ppm)	Target dose (mg/kg bw/day)	Effective daily dose (mg/kg bw/day)	Percentage of target dose (%)
0	0	0	—
7	1	0.9	90
23	3.3	2.8	85
69	10	8.6	86
229	33	17.6	53
694	100	29.8	30
1388	200 (melamine)	123.7	62
1388	200 (cyanuric acid)	167.5	84

Significant treatment-related trends in body weights were observed at day 3 in male and female rats and at days 5, 6, and 7 in male rats. Pairwise comparisons to the control rats indicated significant decreases in body weights at day 3 in male and female rats administered 694 ppm of melamine and cyanuric acid in combination and at days 6 and 7 in male rats given 229 ppm of melamine and cyanuric acid in combination. Given the pronounced decrease in body weights in rats administered 694 ppm of melamine and cyanuric acid, these animals were sacrificed on the third day of treatment.

Macroscopic Evaluation of the Kidneys

Upon sacrifice of the animals, the kidneys were evaluated macroscopically. The kidneys of the two highest combined melamine and cyanuric treatment groups (229 and 694 ppm) were notably enlarged and presented a paler color than the kidneys of the animals from the remaining groups (Supplementary fig. 2S). Prominent depositions of golden brown crystalline structures were evident both in the cortical and the medullary regions of the kidney.

Figure 1 depicts the mean kidney weights (left and right kidneys) determined for each treatment group. In both male and female rats, the weights of the kidneys from the two highest combined treatment groups (229 and 694 ppm) were significantly greater than the ones of the corresponding control animals. No significant differences in kidney weights were detected in the other combined treatment groups or in rats treated with melamine or cyanuric acid alone.

Histopathological Evaluation of the Kidneys

Wet-mount sections from the frozen kidneys revealed golden brown crystals present in renal tubules of all 12 rats in the 229- and 694-ppm combination treatment groups (Fig. 2). The

FIG. 1. Kidney weights of male (gray bars) and female (black bars) F344 rats fed, *ad libitum*, with NIH-41 irradiated feed fortified with 0 (control), 7, 23, 69, 229, or 694 ppm of melamine and cyanuric acid; 1388 ppm of melamine; or 1388 ppm of cyanuric acid. The error bars represent the SEM for each dose group ($n = 6$ per sex). Asterisks denote a significant difference ($p < 0.05$) compared with the control group.

FIG. 2. Representative wet-mount preparations of a kidney from a control rat (panel A) and a rat from the second highest combined melamine and cyanuric acid dose group (229 ppm; panel B). Extensive golden brown crystalline spherulites (black arrows; panel B higher magnification inset) are evident in the kidney of the melamine- and cyanuric acid-treated rat. Scattered crystals were noted in 5 of the 12 rats from the 200 mg/kg melamine-only group (panel C). The morphology of these crystal spherulites (panel C higher magnification inset) was similar to those found in the melamine- and cyanuric acid-dosed animals, but they were much less abundant. The composition of these crystals is being investigated. All panels are of same magnification.

median crystal score for both treatment groups was 4, indicating that most animals developed a large number of crystals in all areas of the kidney (Table 2). Scattered crystals were noted in 5 of the 12 rats treated with melamine only. The morphology of the crystals was similar to those of the animals treated with both melamine and cyanuric acid, but their numbers were greatly reduced. Control animals and those treated with cyanuric acid only had normal appearing sections.

Kidney changes were prominent in the 229- and 694-ppm combination groups of both sexes and featured multiple tubular lesions, including degeneration, necrosis, dilatation, and regeneration along with inflammatory cell infiltrates. The incidence of crystal formation within renal tubules and collecting ducts was 100% in these groups. Unlike the wet-mount sections, no crystals were observed in H&E sections of the melamine-only group of animals. There were no crystals or tubular changes in the control or cyanuric acid-only groups (Supplementary fig. 3S).

Presence of Uroliths and Crystals in the Urinary Bladder

The presence of uroliths and crystals in the urinary bladder was assessed in the bladder lavage fluid of each animal by bright field and polarized light microscopy. The occurrence of round golden brown crystals with radial striations was evident in the majority of the lavages obtained from the animals of the two highest combined treatment groups (229 and 694 ppm), but not in any of the other combined treatment or individual melamine or cyanuric acid groups. No grossly visible stones were present in any of the animals.

Clinical Chemistry

Terminal blood obtained by cardiac puncture was analyzed for BUN and creatinine as markers of the kidney function (Fig. 3). A statistically significant increase in BUN and creatinine was observed in the blood of both male and female rats of the two highest combined treatment groups (229 and 694 ppm) when compared with the control animals. No significant changes were

TABLE 2
Crystal Intensity Observed in Kidney Wet-Mount Sections

Concentration of melamine and cyanuric acid in diet (ppm)	Crystal intensity: males	Crystal intensity: females	Total # of animals with crystals
0	0,0,0,0,0	0,0,0,0,0	
7	0,0,0,0,0	0,0,0,0,0	
23	0,0,0,0,0	0,0,0,0,0	
69	0,0,0,0,0	0,0,0,0,0	
229	4,4,4,4,5	2,3,3,3,4,4	12
694	3,3,4,4,4,4	3,4,4,4,4,4	12
1388 (melamine)	0,0,0,1,2,2	0,0,0,0,2,2	5
1388 (cyanuric acid)	0,0,0,0,0,0	0,0,0,0,0,0	

Note. Presence of crystals was scored on a subjective scale from 0 to 5, with 0 = no crystals seen, 1 = one crystal in entire section, 2 = few crystals with a scattered distribution, 3 = moderate numbers of crystals seen throughout the section, 4 = large numbers of crystals seen immediately when viewing under a microscope, and 5 = extensive numbers of crystals obliterating the regular architecture of kidney.

observed between the other dosed treatment groups and control animals.

DISCUSSION

The 2007 pet food contamination events in the United States and subsequent baby formula contamination in China raised significant concerns regarding the safety of melamine and derivatives (WHO, 2009). The unexpected toxicological synergy and the unavailability of no observed adverse effect level (NOAEL) data for the combined exposure to melamine and cyanuric acid increased the degree of uncertainty in the development of risk assessments for melamine and derivatives and were recognized as key points to be addressed by panels of experts summoned by the World Health Organization (WHO, 2009), the European Food Safety Authority (EFSA, 2010), and the FDA (USFDA, 2008a). A number of studies has been published subsequently to address the combined toxicity of melamine and cyanuric acid; however, the published data are still relatively limited with regard to the exposure time, range of doses tested, and number or sex of test animals (Chen *et al.*, 2009; Dobson *et al.*, 2008; Kim *et al.*, 2010; Kobayashi *et al.*, 2010; Puschner *et al.*, 2007; Reimschuessel *et al.*, 2008, 2010b). In order to expand the available data and clarify further the dose dependency of the nephrotoxic potential of melamine and cyanuric acid, male and female F344 rats were fed *ad libitum* for 7 days with feed fortified with ascending doses of melamine and cyanuric acid or with melamine or cyanuric acid. The range of doses for the combined treatments was selected by considering the data available in the literature at the initiation of the study. These data indicated that in Sprague-Dawley rats, a gavage administration of 400 mg/kg bw/day each of melamine and cyanuric acid led to acute kidney toxicity

FIG. 3. BUN (panel A) and creatinine (panel B) levels determined in the terminal blood of male (gray bars) and female (black bars) F344 rats fed, *ad libitum*, NIH-41 irradiated feed fortified with 0 (control), 7, 23, 69, 229, or 694 ppm of melamine and cyanuric acid; 1388 ppm of melamine; or 1388 ppm of cyanuric acid. The error bars represent the SEM for each dose group ($n = 6$ per sex). Asterisks denote a significant difference ($p < 0.05$) compared with the control group.

in 3 days (Dobson *et al.*, 2008) and that a cat fed 32 mg/kg bw each of melamine and cyanuric acid presented clear signs of illness 24 h after exposure (Puschner *et al.*, 2007). Based upon these data, we hypothesized that a target high dose of 100 mg/kg bw/day each of melamine and cyanuric acid would produce a nephrotoxic response and constitute a positive control for the assay. The remaining combined target doses (1, 3.3, 10, and 33 mg/kg bw/day) were selected in order to establish an adequate dose separation between the high dose and control (0 mg/kg bw/day). In addition, in order to confirm further the synergistic nephrotoxic effect of melamine and cyanuric acid, two separate groups of animals were treated with feed fortified only with melamine or cyanuric acid, aiming to provide a target dose of 200 mg/kg bw/day.

Considering the *ad libitum* nature of this feed study, the effective exposures of each group were determined by taking into account the concentrations of melamine and cyanuric acid in each batch of dosed feed and the feed consumption and body weight recorded daily for each animal. Although no significant differences were observed in the exposure of males versus

females, the higher dosed formulations of combined melamine and cyanuric acid had a marked negative impact on feed consumption, leading to exposures as low as 30% of the target amount (Table 1). The most likely cause of the lower feed consumption in the higher combined melamine- and cyanuric acid-dosed groups was their profound clinical renal failure. There may, however, also have been some palatability issues because lower feed consumption was observed in the melamine-treated animals (ca. 62% of expected, Table 1), where no significant toxic effects were observed. Reduced feed consumption during melamine administration was also noted in a previous study (Melnick *et al.*, 1984). As expected, the significantly reduced feed consumption was accompanied by a drastic weight loss in the highest combined melamine and cyanuric acid group (694 ppm, Supplementary fig. 1S). In addition, a tendency for body weight stagnation was observed in the male animals and a reduced rate of weight gain occurred in the female animals in the second highest combined melamine and cyanuric acid dose group (229 ppm) compared with the control groups. No significant changes were observed in the body weights of the remaining groups treated with combined melamine and cyanuric acid or with melamine or cyanuric acid alone versus the control groups.

Upon sacrifice and necropsy of the animals, it became apparent that the observed changes in body weights constituted a good indicator of the general effects of the treatment, with the animals of the two highest combined dose treatment groups presenting enlarged and paler kidneys than the organs of the remaining animals (Supplementary fig. 2S). Consistently, the kidney weights of these animals were significantly increased (Fig. 1). An examination of longitudinal sections of the kidneys revealed the presence of golden brown crystalline deposits in the cortex and medullary regions of the kidneys from the two highest combined treatment groups. These observations are consistent with a deposition of melamine cyanurate crystals in the kidneys as originally reported in animals coexposed to melamine and cyanuric acid (Dobson *et al.*, 2008; Puschner *et al.*, 2007). Histopathological analysis of the remaining organs failed to indicate any other effects ascribable to the treatments.

To assess better the level of exposure to melamine and cyanuric acid capable of eliciting nephrotoxicity, the kidney tissue of each animal was analyzed by bright field and polarized light microscopy. Given the known solubility of melamine cyanurate in formalin (Reimschuessel *et al.*, 2008) and in order to minimize the possible loss of melamine cyanurate crystals, kidney tissue slides were prepared both by a wet-mount procedure and by a short-term fixation with NBF followed by staining with H&E. Wet-mount analysis of the kidneys from the two highest combined treatment groups revealed the deposition of large numbers of melamine cyanurate-like golden brown birefringent crystalline spherulites, presenting radial symmetry, and often arranged in clusters (Fig. 2, panel B). These crystals were comparable to those reported in previous studies (Dobson *et al.*, 2008; Puschner *et al.*, 2007;

Reimschuessel *et al.*, 2008). Interestingly, crystalline structures were also observable in the wet-mount preparations of the kidneys of 5 of the 12 animals treated with melamine alone (three males and two females), albeit in lower numbers than in the combined treatment groups (Fig. 2, panel C). Limited numbers of crystals have also been reported in kidneys of fish and pigs treated with only melamine (Reimschuessel *et al.*, 2009, 2010a; Puschner *et al.*, 2010). The nature of these crystals is currently under investigation.

The observations conducted on the fixed and stained kidney sections revealed prominent histopathological changes in the two highest combined treatment groups (Supplementary fig. 3S) where both sexes presented renal tubular lesions that included degeneration, necrosis, dilatation, and regeneration accompanied by chronic-active inflammation. Crystals were found in many of the renal tubules and collecting ducts of each rat in these groups, as assessed in both the wet-mount and fixed tissue. Conversely, no crystal deposition or any other histopathological changes were observed in the animals of the remaining groups, including the melamine-only dosed animals. These findings highlight the need to examine unfixed tissue for the presence of crystals, as this technique is more sensitive than evaluation of only H&E sections. The histopathological observations correlated well with the detection of melamine cyanurate-like crystals in the urinary bladder lavaged fluids and the elevated BUN and creatinine levels of the animals from the two highest dose combined treatment groups (Fig. 3).

Four risk assessments have been published pertaining to dietary exposure to melamine and its analogues. In 2008, the FDA established a tolerable daily intake (TDI) of 630 µg/kg bw/day for melamine and its analogues based upon an NOAEL of 63 mg/kg bw/day for the formation of bladder stones in a 13-week rat study with rats administered melamine and applying a 100-fold safety factor (USFDA, 2008b). This was subsequently revised to 63 µg/kg bw/day by increasing the safety factor to 1000-fold (USFDA, 2008a). In 2009, the WHO published a TDI for melamine of 200 µg/kg bw/day (WHO, 2009). This value was derived from benchmark dose (BMD) modeling and was based upon the lower limit of a benchmark dose (BMDL₁₀) of 35 mg/kg bw/day using data from a 13-week rat study with melamine and applying a 200-fold safety (uncertainty) factor. More recently, the EFSA reported a TDI for melamine of 200 µg/kg bw/day (EFSA, 2010). This value was based upon the determination of a BMDL₁₀ of 19 mg/kg bw/day, again using data from a 13-week study with melamine and applying a 100-fold safety factor.

In the current study, an NOAEL of 8.6 mg/kg bw/day was established based upon the formation of crystals in the kidney (Table 2) and the presence of statistically significant increases in kidney weight (Fig. 1), BUN (Fig. 3A), and creatinine (Fig. 3B) at a dose of 17.8 mg/kg bw/day. BMD modeling was also conducted on terminal body weights, kidney weights, BUN, and creatinine (Table 3). Kidney weights were the most sensitive of the endpoints modeled and afforded BMDL's of 8.4–10.9 mg/kg

TABLE 3
Results of Benchmark Modeling of Terminal Body Weights, Kidney Weights, BUN, and Creatinine

Endpoint	Model	AIC ^a	GOF ^b	BMD	BMDL
Terminal body weight	Hill	525.0	0.999	18.1	13.4
	Linear	524.9	0.754	19.2	14.0
	Polynomial	526.1	0.771	22.5	13.7
	Power	526.0	0.802	22.2	14.7
Kidney weight	Hill	793.0	0.942	14.4	10.9
	Polynomial	795.9	0.174	12.1	8.4
	Power	795.2	0.236	11.8	8.8
BUN	Hill	567.8	0.999	17.8	15.8
	Polynomial	573.4	0.132	17.6	15.7
	Power	567.8	0.997	18.2	15.8
Creatinine	Hill	−20.3	0.902	18.6	16.4
	Power	−22.3	0.977	18.6	16.4

Note. Benchmark doses (BMD) and the lower (BMDL) 95% confidence limits were calculated using Environmental Protection Agency Benchmark Dose Software (version 2.1.1; <http://www.epa.gov/ncea/bmds>). The calculations were conducted using Hill, linear, polynomial, and power models to fit the terminal body weights, kidney weights, BUN, and creatinine (mean ± SD) and the mean doses of melamine and cyanuric acid from the entire 7-day study. The BMD was defined as the dose corresponding to a change in the mean response equal to one control SD from the control mean.

^aAIC, Akaike information criterion.

^bGOF, goodness of fit.

bw/day depending upon the specific model. Using the NOAEL and BMDL values leads to TDIs of 8–10 µg/kg bw/day when applying a 1000-fold safety factor, 40–50 µg/kg bw/day when applying a 200-fold safety factor, and 80–100 µg/kg bw/day when applying a 100-fold safety factor. The TDIs calculated from the data in this study are two- to sevenfold lower than previously published TDIs and suggest that TDIs based upon studies conducted with melamine alone may underestimate the risk from coexposures to melamine and cyanuric acid.

SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

FUNDING

National Center for Toxicological Research/Food and Drug Administration (Interagency Agreement No. 224-07-0007) with the National Institute for Environmental Health Sciences/National Toxicology Program.

ACKNOWLEDGMENTS

We are indebted to Dr G. P. Daston (The Procter & Gamble Company, Cincinnati, OH) and to Mona I. Churchwell (NCTR)

for enlightening discussions during the design stage of this work. The opinions expressed in this paper do not necessarily represent those of the USFDA.

REFERENCES

- Chen, K. C., Liao, C. W., Cheng, F. P., Chou, C. C., Chang, S. C., Wu, J. H., Zen, J. M., Chen, Y. T., and Liao, J. W. (2009). Evaluation of subchronic toxicity of pet food contaminated with melamine and cyanuric acid in rats. *Toxicol. Pathol.* **37**, 959–968.
- Crews, G. M., Ripperger, W., Kersebohm, D. B., Güthner, T., and Mertschenk, B. (2006). Melamine and guanamines. In *Ullmann's Encyclopedia of Industrial Chemistry*, 6th ed., pp. 205–221. Wiley-VCH Verlag GmbH & Co. KGaA., Weinheim, Germany.
- Dobson, R. L., Motlagh, S., Quijano, M., Cambron, R. T., Baker, T. R., Pullen, A. M., Regg, B. T., Bigalow-Kern, A. S., Vennard, T., Fix, A., *et al.* (2008). Identification and characterization of toxicity of contaminants in pet food leading to an outbreak of renal toxicity in cats and dogs. *Toxicol. Sci.* **106**, 251–262.
- European Food Safety Authority (EFSA). (2010). *European Food Safety Authority Scientific Opinion on Melamine in Food and Feed (EFSA-Q-2009-00234)*. Available at: <http://www.efsa.europa.eu/en/scdocs/scdoc/1573.htm>. Accessed September 21, 2010.
- Hammond, B. G., Barbee, S. J., Inoue, T., Ishida, N., Levinskas, G. J., Stevens, M. W., Wheeler, A. G., and Cascieri, T. (1986). A review of toxicology studies on cyanurate and its chlorinated derivatives. *Environ. Health Perspect.* **69**, 287–292.
- Huthmacher, K., and Most, D. (2003). Cyanuric acid and cyanuric chloride. In *Ullmann's Encyclopedia of Industrial Chemistry*. pp. 229–250. Wiley-VCH Verlag GmbH & Co. KGaA., Weinheim, Germany.
- Kim, C. W., Yun, J. W., Bae, I. H., Lee, J. S., Kang, H. J., Joo, K. M., Jeong, H. J., Chung, J. H., Park, Y. H., and Lim, K. M. (2010). Determination of spatial distribution of melamine-cyanuric acid crystals in rat kidney tissue by histology and imaging matrix-assisted laser desorption/ionization quadrupole time-of-flight mass spectrometry. *Chem. Res. Toxicol.* **23**, 220–227.
- Kobayashi, T., Okada, A., Fujii, Y., Niimi, K., Hamamoto, S., Yasui, T., Tozawa, K., and Kohri, K. (2010). The mechanism of renal stone formation and renal failure induced by administration of melamine and cyanuric acid. *Urol. Res.* **38**, 117–125.
- Melnick, R. L., Boorman, G. A., Haseman, J. K., Montali, R. J., and Huff, J. (1984). Urolithiasis and bladder carcinogenicity of melamine in rodents. *Toxicol. Appl. Pharmacol.* **72**, 292–303.
- National Toxicology Program (NTP). (1983). Carcinogenesis bioassay of melamine (CAS No. 108-78-1) in F344/N rats and B6C3F1 mice (feed study). United States Department of Health and Human Services, Public Health Service, National Institutes of Health, National Toxicology Program (NTP Technical Report Series No. 245; NTP-81-86; NIH Publication No. 83-2501). Available at: http://ntp.niehs.nih.gov/ntp/htdocs/LT_rpts/tr245.pdf. Accessed September 21, 2010.
- Organisation for Economic Co-operation (OECD). (1998). Organisation for Economic Co-operation and Development Screening Information Dataset (SIDS) for melamine. CAS No. 108-78-1. Available at: <http://www.chem.unep.ch/irptc/sids/OECD/SIDS/108781.pdf>. Accessed September 21, 2010.
- OECD. (1999). Organisation for Economic Co-operation and Development Screening Information Dataset (SIDS) for isocyanuric acid. CAS No. 108-80-5. Available at: <http://www.inchem.org/documents/sids/sids/108805.pdf>. Accessed September 21, 2010.
- Puschner, B., Poppenga, R. H., Lowenstine, L. J., Filigenzi, M. S., and Pesavento, P. A. (2007). Assessment of melamine and cyanuric acid toxicity in cats. *J. Vet. Diagn. Invest.* **19**, 616–624.
- Puschner, B., Reimschuessel, R., and Stine, C. B. (2010). Melamine contamination and pathology. In *Programs and abstracts of the 147th American Veterinary Medical Association Annual Convention*. American Veterinary Association, Atlanta, GA.
- Reimschuessel, R., Evans, E., Andersen, W. C., Karbiwnyk, C. M., Turnipseed, S. B., Mayer, T. D., Nochetto, C., Rummel, N. G., and Giesecker, C. M. (2010a). Residue depletion of melamine and cyanuric acid in catfish and rainbow trout following oral administration. *J. Vet. Pharmacol. Ther.* **33**, 172–182.
- Reimschuessel, R., Evans, E. R., Stine, C. B., Hasbrouck, N., Mayer, T. D., Nochetto, C., and Giesecker, C. M. (2010b). Renal crystal formation after combined or sequential oral administration of melamine and cyanuric acid. *Food Chem. Toxicol.* **48**, 2898–2906.
- Reimschuessel, R., Evans, E. R., Stine, C. B., Stine, C., Mayer, T., Hasbrouck, N., and Giesecker, C. M. (2009). Fish—a non-mammalian model for determining a “No Observable Effect Level” (NOEL) for crystal formation in kidneys after exposure to melamine and cyanuric acid. In *Programs and Abstracts of the 238th National Meeting of the American Chemical Society*. American Chemical Society, Washington, D.C.
- Reimschuessel, R., Giesecker, C. M., Miller, R. A., Ward, J., Boehmer, J., Rummel, N., Heller, D. N., Nochetto, C., de Alwis, G. K., Battaller, N., *et al.* (2008). Evaluation of the renal effects of experimental feeding of melamine and cyanuric acid to fish and pigs. *Am. J. Vet. Res.* **69**, 1217–1228.
- University of Guelph. (2007). *Laboratory Services Division Urgent Bulletins—Pet Food Recall*. Available at: <http://www.labservices.uoguelph.ca/urgent.cfm>. Accessed September 21, 2010.
- U.S. Food and Drug Administration (USFDA). (2008a). *US Food and Drug Administration Interim Safety and Risk Assessment of Melamine and its Analogues in Food for Humans*. Available at: <http://www.fda.gov/Food/FoodSafety/FoodContaminantsAdulteration/ChemicalContaminants/Melamine/ucm164522.htm>. Accessed September 21, 2010.
- U.S. Food and Drug Administration (USFDA). (2008b). *US Food and Drug Administration Interim Safety and Risk Assessment of Melamine and its Analogues in Food for Humans November 28, 2008*; Available at: <http://www.fda.gov/Food/FoodSafety/FoodContaminantsAdulteration/ChemicalContaminants/Melamine/ucm164520.htm>. Accessed September 21, 2010.
- World Health Organization (WHO). (2009). Toxicological and Health Aspects of Melamine and Cyanuric Acid. Report of a WHO Expert Meeting In collaboration with FAO Supported by Health Canada. Ottawa, Canada 1–4 December 2008. World Health Organization, Geneva, Switzerland. Available at: http://whqlibdoc.who.int/publications/2009/9789241597951_eng.pdf. Accessed September 21, 2010.
- Xie, G., Zheng, X., Qi, X., Cao, Y., Chi, Y., Su, M., Ni, Y., Qiu, Y., Liu, Y., and Li, H., *et al.* (2010). Metabonomic evaluation of melamine-induced acute renal toxicity in rats. *J. Proteome Res.* **9**, 125–133.