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Dose-response assessment of nephrotoxicity from a twenty-eight-day combined-exposure to melamine and cyanuric acid in F344 rats

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Abstract

The adulteration of pet food with melamine and derivatives, including cyanuric acid, has been implicated in the kidney failure and death of cats and dogs in the USA and other countries. In a previous 7-day dietary study in F344 rats, we established a no-observed-adverse-effect level (NOAEL) for a co-exposure to melamine and cyanuric acid of 8.6 mg/kg bw/day of each compound, and a benchmark dose lower confidence limit (BMDL) of 8.4–10.9 mg/kg bw/day of each compound. To ascertain the role played by the duration of exposure, we treated F344 rats for 28 days. Groups of male and female rats were fed diet containing 0 (control), 30, 60, 120, 180, 240, or 360 ppm of both melamine and cyanuric acid. The lowest dose that produced histopathological alterations in the kidney was 120 ppm, versus 229 ppm in the 7-day study. Wet-mount analysis of kidney sections demonstrated the formation of melamine cyanurate spherulites in one male and two female rats at the 60 ppm dose and in one female rat at the 30 ppm dose, establishing a NOAEL of 2.1 mg/kg bw/day for males and <2.6 mg/kg bw/day for females, and BMDL values as low as 1.6 mg/kg bw/day for both sexes. These data demonstrate that the length of exposure is an important component in the threshold of toxicity from a co-exposure to these compounds and suggest that the current risk assessments based on exposures to melamine alone may not reflect sufficiently the risk of a co-exposure to melamine and cyanuric acid.

Keywords

Melamine; cyanuric acid; melamine cyanurate; nephrotoxicity

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INTRODUCTION

The intentional adulteration of pet food ingredients with “scrap melamine”, containing melamine and a number of derivatives, including cyanuric acid, has been implicated in the kidney failure and death of a large number of cats and dogs in the US (USFDA, 2007). Other reports of illness and death of cats, dogs, and pigs ascribable to the ingestion of feed contaminated with melamine and derivatives have been made in Europe, South Africa, and Asia (reviewed in Puschner and Reimschuessel, 2011). While melamine and cyanuric acid, high-production-volume chemicals used in a range of industrial and household products, individually present low toxicity (NTP, 1983; Hammond et al., 1986), recent studies revealed that upon simultaneous absorption and systemic distribution, melamine and cyanuric acid can co-crystallize in the nephron as extremely insoluble melamine cyanurate spherulites (Brown et al., 2007; Puschner et al., 2007; Dobson et al., 2008). The accumulation of these spherulites can eventually lead to the loss of renal function and death.

Given the widespread use of products derived from or incorporating melamine or cyanuric acid, trace-to-high levels of these compounds are often detectable in sources as diverse as food contact materials and swimming pool water (WHO, 2008). For example, a total daily exposure of melamine in the range of 30–230 µg/kg bw (body weight) from food contact materials has been estimated for humans living in a set of 12 European countries (EFSA, 2010), and ingestion of cyanuric acid in swimming pool water by long-distance swimmers has been reported to be in the range of 57–397 µg/kg bw (Allen et al., 1982). Thus, an accurate definition of the thresholds of co-exposure capable of eliciting nephrotoxicity and the resulting tolerable daily intake (TDI) values constitutes a crucial step in conducting a science-based risk assessment. In the sequence of the pet food contamination events in the US and later events in China, where infant formula was contaminated with melamine, a number of national regulatory agencies and the World Health Organization (WHO), in collaboration with the Food and Agriculture Organization (FAO), issued risk assessments based on the then available data (WHO, 2008, 2010; USFDA, 2008a, 2008b; EFSA, 2010). However, these agencies recognized that the lack of toxicological data on the combined exposure to melamine and cyanuric acid hampered a proper evaluation of the risks associated with an exposure to the mixture. In a previous short-term study with F344 rats, we determined that the NOAEL for a co-exposure to melamine and cyanuric acid in feed was 8.6 mg/kg bw/day of each compound, and derived a BMDL of 8.4–10.9 mg/kg bw/day of each compound over a period of seven days (Jacob et al., 2011). In contrast, the same length of exposure to substantially higher levels of melamine or cyanuric acid alone failed to produce any significant evidence of nephrotoxicity. These data were consistent with previous reports in the literature demonstrating that a combined exposure to melamine and cyanuric acid was substantially more toxic than that to the individual compounds in cats (Puschner et al., 2007) or rats (Dobson et al., 2008) and later confirmed in a range of mammalian and non-mammalian species (reviewed in Puschner and Reimschuessel, 2011). An important factor to take into consideration when identifying thresholds of toxicity is the role played by the overall duration of exposure to the toxicants. This assessment is particularly important in the toxicological evaluation of toxicants like melamine and cyanuric acid that, due to their ubiquitous nature, may provide more or less continuous low-levels of exposure in animals and humans. In order to ascertain the role played by the duration of exposure in the dose-response to a combination of melamine and cyanuric acid, we have now treated F344 rats under conditions comparable to those of our previous seven-day study, but extended the treatment period to 28 days. We report herein the outcome of this study, and discuss its results in light of the data currently available in the literature and currently existing risk assessments.

MATERIALS AND METHODS

Chemicals

Melamine (Aldrich, stated purity 99%, CAS 108-78-1) and cyanuric acid (Fluka, anhydrous, stated purity > 98%) were obtained from Sigma-Aldrich, St. Louis, MO. The purity and identity of the test articles were confirmed by high performance 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, St. Louis, MO, and met or exceeded American Chemical Society specifications.

Preparation of the dosed feed

The melamine and cyanuric acid were individually pulverized to fine powders in a mechanical ball mill (Spex Industries 8000 mixer/mill, Metuchen, NJ) 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), 30, 60, 120, 180, 240, or 360 ppm each of melamine and 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 data for 10-week-old male and female F344 rats and aimed to afford the exposures of, respectively, 0, 2.5, 5, 10, 15, 20, or 30 mg/kg bw/day each of melamine and cyanuric acid. The control feed underwent the same mechanical blending procedure as the dosed feed preparations.

Dosed feed certification

The dosed feed formulations were analyzed by isotopic dilution mass spectrometry as previously described (Jacob et al., 2011). The analyses revealed that the melamine and cyanuric acid in the feed formulations were on average, respectively, at $100.0 \pm 1.6\%$ and $100.6 \pm 2.9\%$ of the target concentrations. The homogeneity error of the formulations, as assessed by the relative standard deviation of nine independent feed formulation samples drawn from the top, middle, and bottom of the feed blender used in the formulations, was 3.8% in all cases.

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 (12 males and 12 females per dose group, 10-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 28 days with NIH-41 irradiated meal (control animals) or with NIH-41 irradiated meal containing melamine and cyanuric acid (treatment 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% with 10–15 air changes per hour, a temperature of $22 \pm 4^\circ\text{C}$, and a 12-hour light/day cycle. Food consumption and body weights were measured daily.

Histopathological procedures

At the end of the 28-day exposure period, the animals were anesthetized by carbon dioxide inhalation and blood was withdrawn by cardiac puncture until exsanguination. All tissues listed in the National Toxicology Program (NTP) specifications for histopathology in 28-day studies (NTP, 2011) were examined grossly, removed, and preserved in 10% neutral buffered formalin (NBF), with the exception of the eyes and testes, which were fixed in modified Davidson's fixative. In addition, the following special procedures were conducted

on the kidneys: Left kidney - sectioned longitudinally; one half was flash frozen for wet-mount analysis; the other half was sectioned transversally, ¼ of the kidney was fixed in NBF for 2 hours, and the other ¼ was fixed in 70% ethanol. Right kidney - sectioned transversally; one half was frozen for residue analysis; the other half was sectioned longitudinally, ¼ of the kidney was fixed in NBF for 2 hours, and the other ¼ was fixed in 70% ethanol. There were no appreciable differences between the two fixation methods. Only data from the NBF fixation are reported here. The fixed tissues were trimmed, processed, and embedded in Formula R, sectioned at approximately 5 µm, and stained with hematoxylin and eosin (H&E). Lesions were graded for severity as 1 (minimal), 2 (mild), 3 (moderate), or 4 (marked).

Wet-mount procedures

The wet-mount analyses were conducted by pressing approximately 2-mm-thick sections (approximately 50–100 mg) of thawed flash-frozen tissue between two microscope slides and observation under bright field and polarized light microscopy. The presence of crystals was scored on a subjective scale from 0 – 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.

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.

Hematology

Terminal blood samples obtained by cardiac puncture were collected on tubes with EDTA and the analyses were performed on the same day of collection. Complete blood counts were determined on an ABX Pentra 60 C+ analyzer (ABX, Irvine, CA) using ABX reagents. Maintenance and calibration was done according to the manufacturer's recommendations. Three levels of assayed controls were included in daily analyses as internal controls.

Melamine and cyanuric acid residue analysis in the kidneys

Melamine and cyanuric acid residues in the kidneys were quantified by liquid chromatography coupled with isotopic dilution mass spectrometry using a system comprised of an Acquity ultraperformance liquid chromatograph (UPLC) (Waters Corporation, Milford, MA) interfaced with a Waters Quattro Premier XE tandem mass spectrometer equipped with an electrospray ionization probe. Kidney tissue (*ca.* 200–800 mg) was placed in a 2 mL microcentrifuge tube, fortified with 375 µL of 100 µg/mL solutions (25% methanol in water) of ¹⁵N₃-melamine and ¹³C₃-cyanuric acid (Toronto Research Chemicals Inc, North York, Ontario) and homogenized in a Tissuelizer ball mill (Qiagen, Germantown, MD) with a 5 mm stainless steel sphere at the highest speed setting for 40 minutes. 100 µL of the homogenate thus obtained were added to 900 µL of 2.5% HCl in water, mixed for one hour at room temperature, and centrifuged at 21,000 *g* for 10 minutes. A 10 µL sample of the supernatant thus obtained was added to 990 µL of acetonitrile containing 1% water, the mixture was centrifuged for 10 minutes at 21,000 *g*, and 450 µL of the supernatant thus obtained were transferred to 0.2 µm PTFE Whatman mini-uniprep syringeless autosampler filter vials (Whatman Inc., Piscataway, NJ). For the analysis of low-level samples, 750 µL

of the supernatant were taken to dryness under a nitrogen stream at 75°C and were reconstituted in 75 µL of acetonitrile containing 1% water. 10 µL of each sample were injected in the UPLC and eluted isocratically in a 150 × 2.1 mm ZIC-HILIC column (SeQuant, Umeå, Sweden) at 450 µL/min with 5% of 10 mM ammonium acetate in water in 95% acetonitrile. The mass spectral data acquisition was conducted in multiple reaction monitoring mode essentially as described in Jacob and Gamboa da Costa (2011). Plots of response ratios for labeled vs. unlabeled melamine and cyanuric acid were linear (r^2 0.999) over the concentration range of 2.5–1000 ppb unlabeled melamine and cyanuric acid plus 250 ppb $^{13}\text{C}_3$ -cyanuric acid and 250 ppb $^{15}\text{N}_3$ -melamine. The methodology was validated by analyzing kidney tissue samples obtained from untreated rats spiked with 10, 1000, or 5000 µg/g of melamine cyanurate on two separate days ($n = 5$ for each concentration per day), affording intra- and inter-day accuracies of 99.8–103.7% and imprecision 3.1%. The lower limit of quantification was estimated at 25 ppb of melamine in kidney tissue, with an imprecision of 5.1% and an accuracy of 109.6% ($n = 4$), and at 250 ppb of cyanuric acid, with an imprecision of 1.9% and an accuracy of 110.1% ($n = 4$).

Statistics

Body weight 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 Tukey's Studentized Range test.

Kidney weights and clinical chemistry and hematology measurements were analyzed by one-way analysis of variance (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. In instances where there was still an unsatisfactory data distribution or variance, the data were analyzed by a Kruskal-Wallis one-way ANOVA on Ranks, with pairwise comparisons to the control group conducted by Dunn's method.

Histopathology results were analyzed by a one-tailed Fisher's Exact test. 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>). Hill and exponential models (EFSA, 2009) were used to fit continuous variables (the mean ± standard deviation of kidney weights, BUN, creatinine, and kidney levels of melamine and cyanuric acid) and the amount of melamine and cyanuric acid consumed during the first day of the study. The BMD for continuous variables, except the kidney levels of melamine and cyanuric acid, was defined as the dose corresponding to a change in the mean response equal to one control standard deviation from the control mean. For the kidney levels of melamine and cyanuric acid, the BMD was defined as the dose corresponding to a change in the mean response equal to one control standard deviation from the mean lowest administered dose of melamine or cyanuric acid. Gamma, logistic, log-logistic, log-probit, multistage, probit, and Weibull models were used to fit dichotomous variables (kidney crystals in H&E-stained slides, renal tubule dilatation, renal tubule epithelium necrosis, renal polymorphonuclear cellular infiltration, renal lymphocyte cellular infiltration, pelvis transitional epithelium hyperplasia, and kidney crystals determined by wet mount) and the amount of melamine and cyanuric acid consumed during the first day of the study. The BMD for dichotomous variables was defined as the dose corresponding to an excess risk of 10%.

RESULTS

Animal morbidity and mortality

Acute toxicity was observed in the two highest dose groups. All animals in the 360 ppm melamine and cyanuric acid group were removed on days 3–5; 15 of the 24 rats in the 240 ppm melamine and cyanuric acid group were removed between days 4–25 of treatment (median = 16 days). A single male rat in the 180 ppm melamine and cyanuric acid group was removed on day 14. Common symptoms of acute toxicity in these animals were emaciation, abnormal body posture, and hematuria. One female rat in the 120 ppm melamine and cyanuric acid group was removed on day 4 due to evidence of hematuria. The necropsy of this rat revealed the presence of a large stone unrelated to treatment (*ca.* $17 \times 8 \times 7$ mm) in the urinary bladder. A single male rat in the 60 ppm melamine and cyanuric acid group was removed on day 1 of treatment due to abdominal swelling. The necropsy of this animal failed to reveal any cause for the swelling.

Effective exposure

The effective mean daily dose of melamine and 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. Table 1 itemizes the effective exposures to melamine and cyanuric acid in each group. The average effective exposure (mg/kg bw/day) in both sexes was proportional to the dietary concentration (ppm) at doses up to 180 ppm, whereupon the value decreased due to a decrease in food intake. Female rats were exposed on average to *ca.* 30% higher doses than the male rats as a result of a higher daily mean consumption of feed per unit of body weight.

Body and kidney weights

The body weights of the rats were measured in the morning of each day of treatment (Figure 1S, Supplementary Data). Significant treatment-related trends in body weights were observed at days 4 and 14–28 in male rats and at days 3, 4 and 6–28 in female rats. Pairwise comparisons to the control rats indicated significant decreases in body weights at day 4 in male rats and days 3 and 4 in female rats administered the highest level (360 ppm) of melamine and cyanuric acid, and at days 14–28 in male rats and days 6–28 in female rats administered the next to highest dose (240 ppm) of melamine and cyanuric acid. The reduction of body weight assessed on the day of necropsy in the 240 and 360 ppm groups in relation to the respective controls was approximately 23% in the females, and 30% in the males. Male and female rats administered the two highest doses of melamine and cyanuric acid (240 and 360 ppm) had significant increases in kidney weights (Table 1S, Supplementary Data).

Histopathological evaluation of the kidneys

A significant increase in the incidence of crystals consistent with melamine cyanurate was observed in both sexes of rats administered the four highest doses (120, 180, 240, and 360 ppm) of melamine and cyanuric acid (Table 2). Crystals were not observed in the control rats or in the 30 and 60 ppm dose groups. Male rats administered the three highest doses (180, 240, and 360 ppm) of melamine and cyanuric acid had a significant increase in renal tubule dilatation, renal tubule epithelium necrosis, renal polymorphonuclear cellular infiltration, renal lymphocyte cellular infiltration, and pelvis transitional epithelium hyperplasia (Table 2). In female rats, a significant increase in renal tubule dilatation, renal tubule epithelium necrosis, renal lymphocyte cellular infiltration, and pelvis transitional epithelium hyperplasia occurred at the two highest doses (240 and 360 ppm). Renal fibrosis was observed at the 180 and 240 ppm doses in both sexes of rats but not at the highest dose

(360 ppm). The lack of renal fibrosis in the highest dose group may be due to the fact that these rats were removed from the study after only 3–5 days of treatment. Although there were no statistically-significant differences registered in the incidence of renal tubule regeneration, a dose-effect was observed in the severity of the response, presumably reflecting the degree of regenerative response of the organ to the crystal-induced lesions. A full necropsy conducted on all rats failed to reveal the occurrence of treatment-related lesions in any other organ or tissue.

Wet-mount evaluation of the kidneys

Kidneys of the control groups had normal appearing renal tubules. In contrast, wet-mount sections revealed golden brown crystals present in the renal tubules of 100% of the rats exposed to the feed containing 120, 180, 240, or 360 ppm of melamine and cyanuric acid. Even the female rat removed from the study at 4 days in the 120 ppm dose group was already positive for crystal presence. Crystal spherulites were noted in 1 out of 24 rats exposed to 30 ppm and 3 out of 23 rats exposed to 60 ppm of melamine and cyanuric acid. The intensity of the crystal response is shown in Table 3. Generally, higher doses resulted in higher crystal intensity. For example, crystal intensity grades of 4 or 5 were found in 6, 11, 17, and 13 rats exposed to the formulations containing 120, 180, 240, and 360 ppm of melamine and cyanuric acid, respectively. It should be noted that crystals in the 240 ppm dose group had a significant impact in the health of the animals to the point that six of these animals had to be removed from study by day 5, with most being removed by day 17, which could have reduced the number of animals with higher intensity grades. A comparison of male and female intensity grades shows that grades of 1–3 were found in 39% of females compared to 24% of males, while the higher scores (4 and 5) were found in only 21% of the females versus 35% of the males. Thus, overall, it appears that the males tend to have a greater number of crystals observed by wet mount analysis.

Clinical chemistry and hematology

BUN and creatinine levels were significantly increased in both sexes of rats administered the two highest doses (240 and 360 ppm) of melamine and cyanuric acid (Figure 1). Alanine aminotransferase (ALT) was significantly decreased at the two highest doses in both sexes (Table 2S, Supplementary Information). Alkaline phosphatase was significantly decreased at the three highest doses in male rats and at the two highest doses in female rats. Other sporadic changes were observed in clinical chemistry (Table 2S, Supplementary Information) and hematology (Table 3S, Supplementary Information) parameters. The alterations in ALT and alkaline phosphatase, and the other sporadic changes were not considered to be ascribable to the direct toxicity of the combined exposure to melamine and cyanuric acid, but rather to the poor general health condition of the rats given their compromised kidney function and pronounced body weight losses in relation to the control group.

Melamine and cyanuric acid residue analysis in the kidneys

The kidney concentrations of melamine and cyanuric acid were determined by UPLC coupled with isotope dilution tandem mass spectrometry (Figure 2). In both sexes, the samples obtained from the animals fed diet containing 30 or 60 ppm of melamine and cyanuric acid were found to contain detectable, but very low, levels of these triazines (<1 ppm). In the kidney samples obtained from animals fed diet containing 120, 180, or 240 ppm of melamine and cyanuric acid substantially higher values were determined. Although noticeable intragroup differences were apparent, a general dose-response was observed in these treatment groups. In both sexes, the melamine and cyanuric acid levels determined in the highest dose group (360 ppm) were lower than those determined in the next lowest dose group (240 ppm), presumably due to the fact that the 360 ppm animals were removed from

the study after only 3–5 days of treatment. With the exception of the two lowest dose groups, where the quantification imprecision was higher due to the very low levels determined, the molar ratio of melamine to cyanuric acid was *ca.* 1.0–1.1.

BMD calculations

BMD modeling was conducted on kidney weights, BUN, creatinine, crystals in the kidneys, renal tubule dilatation, renal tubule epithelium necrosis, renal polymorphonuclear cellular infiltration, renal lymphocyte cellular infiltration, transitional epithelium hyperplasia of the pelvis, melamine concentration in the kidney, and cyanuric acid concentration in the kidney. Of these endpoints, the most sensitive was crystals in the kidney assessed by wet-mount, with BMDL values of 1.6–3.7 and 1.6–4.1 mg/kg bw/day in males and females, respectively, and crystals in the kidney assessed in the thin H&E-stained kidney sections with BMDL values of 2.1–3.9 mg/kg body weight/day in male rats and 4.9–6.1 mg/kg body weight/day in female rats depending upon the specific model (Table 4S, Supplementary Information). Other endpoints that had low BMDL values included renal lymphocyte cellular infiltration (3.0–3.5 and 6.3–9.6 mg/kg body weight/day in male and female rats, respectively), and the melamine concentration in the kidney (4.7–10.7 and 4.9–10.7 mg/kg body weight/day in male and female rats, respectively).

DISCUSSION

In a previous study we reported the dose-response observed in F344 male and female rats exposed to equal amounts of melamine and cyanuric acid in feed for seven days (Jacob et al., 2011). In order to ascertain the role played by the length of co-exposure to these nephrotoxics we have now extended the duration of the treatment to 28 days. Ten-week-old F344 rats (12 males and 12 females per dose group) were fed *ad libitum* for 28 days with NIH-41 irradiated meal fortified with 0 (control), 30, 60, 120, 180, 240, or 360 ppm each of melamine and cyanuric acid. These concentrations aimed to afford exposures of, respectively, *ca.* 2.5, 5, 10, 15, 20, and 30 mg/kg bw/day of melamine and cyanuric acid. The highest target dose (30 mg/kg bw/day) was selected based on the fact that a comparable target dose (33 mg/kg bw/day) had constituted the lowest dose where noticeable toxicity was observed in our previous seven-day study in this rat strain, and the remaining doses were decreased to a level where no effect was anticipated. As expected, acute toxicity was observed in the highest treatment group (360 ppm) and all animals were removed from the study for humane reasons between days 3–5. Marked toxicity was also observed in some animals in the 240 ppm dose group and only 9 out of 24 rats were kept on study until the scheduled sacrifice date. As a result of the very rapid onset of toxicity and consequent poor general health of the rats in the highest doses, the average daily feed consumption for the duration of exposure was notably affected, resulting in effective daily doses substantially lower than those targeted (Table 1). This effect was particularly pronounced in the highest dose group where the food consumption of both males and females in the first 24 hours of treatment was *ca.* 40% lower than that recorded for the matching controls (data not shown). It is plausible that given the very fast absorption of mixtures of melamine and cyanuric acid in the gastrointestinal tract of rats (Jacob et al., 2012), the high concentrations of these triazines in the feed in the highest dose group may have led to an onset of nephrotoxicity within the first day of exposure. In the remaining dose groups, the effective doses were closer to those targeted; however, the higher daily feed consumption per unit of body weight in the females resulted in doses approximately 30% higher on average than those attained in the male rats.

Consistent with our previous observations in the seven-day exposure study (Jacob et al., 2011) and observations in other previous rodent studies (Dobson et al., 2008; Chen et al., 2009; Choi et al., 2010; Park et al., 2011), significant decreases in the body weight and

increases in the kidney weights were registered in both male and female rats in the two highest treatment dose groups in relation to their matching controls (Figure 1S and Table 1S, Supplementary Information). A detailed histopathological analysis of the kidneys of the male and female rats in the two highest dose groups (240 and 360 ppm) revealed a number of lesions, including an increase in renal tubule dilatation, tubule epithelial necrosis, and pelvis transitional epithelium hyperplasia (Table 2). In line with these observations, and although notable intragroup variations were observed, significant elevations in BUN and creatinine levels were determined in the terminal blood of rats of both sexes in these dose groups in relation to their matched controls (Figure 1). Kidney lesions were also significantly increased in male but not in female rats at the next lowest dose (180 ppm) suggesting that males may be more sensitive than females to the nephrotoxic effects of the combination of melamine and cyanuric acid. It should be noted that although not meeting statistical significance, the observations of lymphocytic cellular infiltration in females at the 120 and 180 ppm groups, and observations of transitional epithelium hyperplasia in females in the 120 and 180 ppm groups and males in the 120 ppm group may reflect a real biologic effect, since these lesions were not observed in any of the corresponding sexes in the 0, 30, or 60 ppm groups (Table 2). The histopathological analysis also revealed the formation of typical golden brown crystalline spherulites (presumably melamine cyanurate) in the kidney tissue of all rats in the two highest dose groups, in 10 of 12 males and 8 of 12 females in the 180 ppm group, and in 10 of 12 males and 6 of 12 females in the 120 ppm group. The formation of melamine cyanurate crystals was also assessed by a wet mount procedure, a technique capable of analyzing substantially larger samples of tissue and avoiding possible crystal losses due to the tissue processing required for the preparation of H&E-stained thin sections (Reimschuessel et al., 2008). As expected, the wet mount analysis corroborated the detection of crystals at the four highest dose groups (Table 3). In addition, the formation of crystals was also evident in one male and two females in the 60 ppm group and in one female in the 30 ppm group. In order to enable a more quantitative assessment of the accumulation of melamine cyanurate crystals in the kidneys, the melamine and cyanuric acid concentrations were analyzed in *ca.* 200–800 mg samples of these kidneys by UPLC coupled with isotope dilution tandem mass spectrometry (Figure 2). A good correspondence was observed between the melamine and cyanuric acid concentrations and the assessment of crystals by wet mount. Although substantial intragroup variability was evidenced by both methodologies, a dose-response was observed in the range of 120 – 240 ppm doses either as assessed by wet mount (Table 3) or mass spectrometry (Figure 2). With the 30 and 60 ppm doses, a good correspondence was also observed, with a sharp decrease in the crystal incidence and intensity observed in the wet mounts (Table 3) and markedly lower (*ca.* 800X; data not shown) concentrations of melamine and cyanuric acid in relation to the 120 ppm group determined by mass spectrometry (Figure 2). We ascribe the lower crystal intensities and concentrations of melamine and cyanuric acid determined in the highest dose group to the early sacrifice of the rats. Importantly, the concentrations of melamine and cyanuric acid in the kidneys presented a molar ratio of melamine to cyanuric acid of *ca.* 1.0 $\approx$ 1.1, a ratio consistent with the 1:1 ratio of melamine to cyanuric acid found in the lattice of melamine cyanurate (Seto and Whitesides, 1990). A comparable observation has been reported in rats treated with melamine and cyanuric acid (Dobson et al., 2008). On average, the concentrations of melamine and cyanuric acid were higher in the male rat kidneys than those in the female rat kidneys (Figure 2), suggesting that the male rats were more susceptible to the accumulation of melamine cyanurate crystals than the females. This observation is consistent with the fact that at the intermediate dose of 180 ppm, histopathological lesions were observed in the male rats, but not in the female rats.

Considering all the parameters analyzed in this study, the wet-mount assessment of crystal formation constituted the most sensitive indicator of adverse effects, with crystals detected in one male and two female rats in the 60 ppm group and in a single female rat in the 30

ppm group. Given the effective experimental doses (Table 1), these observations establish a NOAEL for the male rats at 2.1 mg/kg bw/day, and a NOAEL for female rats under 2.6 mg/kg bw/day. These values compare with a NOAEL of 8.6 mg/kg bw/day in our previous seven-day study for both male and female rats (Jacob et al., 2011). A very limited number of other studies addressing a co-exposure to melamine and cyanuric acid in rodents have been reported in the literature. Among these, only a single study reached or exceeded 28-days of exposure (Chen *et al.*, 2009); in this three-month study, the authors fed groups of rats with pet food contaminated with melamine and cyanuric acid that was blended with variable percentages of regular rat chow and determined NOAEL values of *ca.* 11.8 mg/kg bw/day of cyanuric acid and 80.4 mg/kg bw/day of melamine for male rats and *ca.* 14.6 mg/kg bw/day of cyanuric acid and 99.7 mg/kg bw/day of melamine for female rats. These NOAEL values are substantially higher than those reported in the current study, in particular considering the 3-fold difference in the exposure period. This discrepancy may be partially explained by the fact that the authors apparently did not conduct a wet-mount analysis of the kidney tissue, and fixed the tissues in 10% formalin for one week, possibly leading to the solubilization and loss of most melamine cyanurate crystals. The NOAEL values reported in the present study are, however, comparable with those reported in a 28-day study conducted with weanling pigs, where a NOAEL for a dietary co-exposure for melamine and cyanuric acid was 1.0 mg/kg bw/day (Stine et al., 2011).

Benchmark dose modeling was also conducted for a selected number of the endpoints previously discussed in order to enable a more quantitative approach to the assessment of the dose-response. The wet-mount assessment of melamine cyanurate crystals afforded the lowest BMDL, with values of 1.6–3.7 and 1.6–4.1 mg/kg bw/day for males and females, respectively (Table 4S, Supplementary Data). These values contrast with the BMDL of 8.4–10.9 mg/kg bw/day determined for kidney weights (the most sensitive endpoint) in the seven-day study (Jacob et al., 2011). It is thus clear that in F344 rats the duration of a dietary co-exposure to melamine and cyanuric acid plays a key role in defining the threshold of exposure capable of eliciting nephrotoxicity.

A number of risk assessments for a dietary human exposure to melamine have been published by national and international agencies (US FDA, 2008a, 2008b; EFSA, 2010; WHO, 2008, 2010). Although the particular TDI values derived in these risk assessments varied according to the specific points of departure and safety factors considered, all assessments were based on toxicological data stemming from an NTP-sponsored 13-week rat study conducted with dietary exposure to melamine alone (NTP, 1983; Melnick et al., 1984). In this study the lowest dose (63 mg/kg bw/day) afforded an incidence of bladder stone formation (the most sensitive endpoint in the study) that was not statistically different from that determined in the control rats. The bladder stone incidence data were used to derive a NOAEL of 63 mg/kg bw/day (USFDA, 2008a) and BMDL₁₀ values of 35 mg/kg bw/day (WHO, 2008) or 19 mg/kg bw/day (EFSA, 2010), which constituted the points of departure for the currently existing risk assessments. In the present study NOAEL values of 2.1 mg/kg bw/day for male rats and <2.6 mg/kg bw/day for female rats were established based upon crystal formation in the kidneys, and BMDL values as low as 1.6 mg/kg bw/day were determined for both male and female rats using the same endpoint; hence, our data indicate that the points of departure currently considered in risk assessments for melamine alone may underestimate the toxic potential of a dietary co-exposure to melamine and cyanuric acid by at least a factor of 10

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

A 28-day dietary co-exposure to melamine and cyanuric acid was conducted in F344 rats

The NOAELs were 2.1 mg/kg bw/day for males and <2.6 mg/kg bw/day for females

BMDL values as low as 1.6 mg/kg bw/day for both sexes were determined

The length of exposure plays an important role in the threshold of toxicity

Current assessments may underestimate the risk of melamine and cyanuric acid

Figure 1.

Blood urea nitrogen (BUN) and creatinine concentrations determined in the terminal blood of male and female F344 rats fed *ad libitum* with NIH irradiated feed fortified with 0 (control), 30, 60, 120, 180, 240, or 360 ppm of melamine and cyanuric acid. Asterisks (*) denote a significant difference ($p < 0.05$) compared to the matching control group. $n = 12$, except for male rats exposed to 30 ppm of melamine and cyanuric acid where $n = 11$.

Figure 2.

Concentrations (ng/g) of melamine and cyanuric acid determined in kidney samples of male and female F344 rats fed *ad libitum* with NIH irradiated feed fortified with 0 (control), 30, 60, 120, 180, 240, or 360 ppm of melamine and cyanuric acid. n = 12, except for male rats exposed to 30 ppm of melamine and cyanuric acid where n = 11.

Table 1

Average effective experimental dose of melamine and cyanuric acid for male and female F344 rats during the treatment period.

Concentration of melamine and cyanuric acid in diet (ppm)	Male rats (mg/kg bw/day)	Female rats (mg/kg bw/day)
0	0	0
30	2.1	2.6
60	4.3	5.3
120	8.6	11.2
180	12.3	16.4
240	10.4	15.0
360	8.7	12.1

Table 2

Incidence and severity of histopathological findings in H&E-stained slides from the kidneys of F344 rats fed *ad libitum* with NIH irradiated feed fortified with 0 (control), 30, 60, 120, 180, 240, or 360 ppm of melamine and cyanuric acid.

Concentration of melamine and cyanuric acid in diet (ppm)		0	30	60	120	180	240	360
Crystals	Males	0/12 ^a	0/12	0/12	10/12 [*]	10/12 [*]	12/12 [*]	12/12 [*]
	Females	-	-	-	<0.0001	<0.0001	<0.0001	<0.0001
Renal tubule dilatation	Males	0/12	0/12	0/12	6/12 [*]	8/12 [*]	12/12 [*]	12/12 [*]
	Females	-	-	-	0.0069	0.0007	<0.0001	<0.0001
Fibrosis	Males	0/12	0/12	0/12	0/12	10/12 [*] (2.4) ^b	12/12 [*] (3.4)	12/12 [*] (3.2)
	Females	-	-	-	-	<0.0001	<0.0001	<0.0001
Renal tubule regeneration	Males	0/12	0/12	0/12	0/12	3/12 (2.0)	12/12 [*] (3.5)	12/12 [*] (3.7)
	Females	-	-	-	-	-	<0.0001	<0.0001
Renal tubule epithelium necrosis	Males	0/12	0/12	0/12	0/12	4/12 [*] (1.7)	4/12 [*] (3.2)	0/12
	Females	-	-	-	-	0.0466	0.0466	-
Mineralization	Males	0/12	0/12	0/12	0/12	1/12 (2.0)	6/12 [*] (2.5)	0/12
	Females	-	-	-	-	0.5000	0.0069	-
Renal tubule regeneration	Males	11/12 (1.0)	12/12 (1.0)	11/12 (1.1)	11/12 (1.1)	12/12 (2.9)	12/12 (3.5)	12/12 (3.5)
	Females	7/12 (1.0)	5/12 (1.0)	7/12 (1.0)	6/12 (1.0)	11/12 (1.9)	12/12 (3.5)	12/12 (3.7)
Renal tubule epithelium necrosis	Males	0/12	0/12	0/12	0/12	5/12 [*] (1.0)	8/12 [*] (2.2)	5/12 [*] (1.4)
	Females	-	-	-	-	0.0186	0.0007	0.0186
Mineralization	Males	0/12	0/12	0/12	0/12	0/12	10/12 [*] (1.8)	7/12 [*] (1.1)
	Females	-	-	-	-	-	<0.0001	0.0023
Mineralization	Males	8/12 (1.0)	10/12 (1.1)	8/12 (1.0)	10/12 (1.0)	7/12 (1.4)	3/12 (1.0)	4/12 (1.0)
	Females	-	-	-	-	-	-	-

Concentration of melamine and cyanuric acid in diet (ppm)		0	30	60	120	180	240	360
Polymorphonuclear cellular infiltration	Females	12/12 (3.2)	12/12 (3.1)	12/12 (3.0)	12/12 (3.5)	12/12 (3.4)	12/12 (2.3)	12/12 (2.7)
	Males	0/12	0/12	0/12	0/12	5/12* (1.8)	8/12* (3.2)	7/12* (1.0)
		-	-	-	-	0.0186	0.0007	0.0023
	Females	0/12	0/12	0/12	0/12	0/12	11/12* (2.1)	3/12 (1.0)
Lymphocyte cellular infiltration		-	-	-	-	-	<0.0001	0.1087
	Males	0/12	0/12	1/12	0/12	6/12* (1.3)	4/12* (1.7)	3/12 (1.0)
		-	-	-	-	0.0069	0.0466	0.1087
	Females	0/12	0/12	0/12	1/12 (1.0)	3/12 (1.3)	5/12* (1.6)	6/12* (1.1)
Pelvis, transitional epithelium hyperplasia		-	-	-	0.5000	0.1087	0.0186	0.0069
	Males	0/12	0/12	0/12	2/12 (2.0)	5/12* (2.2)	10/12* (1.8)	12/12* (1.9)
		-	-	-	0.2391	0.0186	<0.0001	<0.0001
	Females	0/12	0/12	0/12	1/12 (2.0)	2/12 (1.5)	11/12* (2.0)	12/12* (1.5)
		-	-	-	0.5000	0.2391	<0.0001	<0.0001

* Incidence significantly different from matching control (P-values indicated in italics);

^aIncidence of lesion

^bSeverity of lesion 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.

Table 3

Number of F344 rats with the given crystal intensity in wet-mount preparations of their kidneys after being fed *ad libitum* with NIH-41 irradiated feed fortified with 0 (control), 30, 60, 120, 180, 240, or 360 ppm of melamine and cyanuric acid.

Concentration of melamine and cyanuric acid in diet (ppm)	Crystal intensity - male rats ^a						
	0	1	2	3	4	5	
0	12						
30	12						
60	9		1				
120			2	6	4		
180			2	3	5	2	
240					3	6	3
360				3	7	2	

Concentration of melamine and cyanuric acid in diet (ppm)	Crystal intensity - female rats ^a						
	0	1	2	3	4	5	
0	12						
30	11		1				
60	10		2				
120			4	6	2		
180			1	7	3	1	
240				4	7	1	
360			3	5	4		

^aThe presence of crystals was scored on a subjective scale from 0 – 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.