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Toxicity of Dietary Melamine to Laying Ducks: Biochemical and Histopathological Changes and Residue in Eggs

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Jinding laying ducks (n=648) were subjected to one of six dietary treatments (0, 1, 5, 25, 50, or 100 mg of melamine/kg of diet) to investigate the toxicity of melamine and determine the melamine residue in eggs. Ducks were fed melamine-supplemented diets for 21 days followed by a 21 day withdrawal period. Dietary melamine had no adverse effects on laying performance. Renal lesions were correlated with increasing levels of dietary melamine. Melamine residue in eggs increased with dietary melamine during the first 21 days and reached the maximum content (1.35 mg/kg) in the 100 mg of melamine/kg of diet group. Melamine residue in eggs decreased rapidly during the withdrawal period. The depletion time for egg melamine residue increased with dietary melamine level. These results indicated that a dietary level of ≥50 mg of melamine/kg of feed induces obvious renal injury. The residue level and withdrawal time for melamine clearance in eggs correlated with the dietary melamine level.

KEYWORDS: Melamine; egg residue; histopathology; kidney; laying duck

INTRODUCTION

Melamine (1,3,5-triazine-2,4,6-triamine) is a synthetic chemical used primarily for the production of amino resins, plastics (*1*−*3*), and fertilizer because of its high nitrogen content (*4*). It is also a minor metabolite of the insecticide cyromazine (*5*, *6*). In 1978, Newton and Utley tried to evaluate melamine as a non-protein nitrogen source for ruminants, but they found that it is difficult for cattle to hydrolyze (*7*). Recently, reports indicating that pet food contaminated with melamine resulted in renal disease and deaths in cats and dogs have placed melamine contamination in the spotlight (*8*, *9*). In the case of the pet food, it has been speculated that melamine was added intentionally in feed for a false high level of crude protein determined by the Kjeldahl method (*10*). Some swine, fish, and poultry feeds were reported to be contaminated with 30−120 mg of melamine/kg of diet (*11*−*13*). Milk products or eggs contaminated with melamine were reported in Hong Kong and mainland China in 2008, which was considered to be a result of the illegal addition of melamine in milk or feed. The concentration of melamine in some infant formulas and fresh eggs was determined to be as high as 2500 and 4.7 mg/kg, respectively (*14*, *15*). Therefore, addition of melamine to feed may contaminate animal products and has raised worldwide concerns about food safety.

A previous study showed that melamine acute toxicity in animals was generally very low (*16*). A dosage of 181 mg of melamine/kg of body weight did not affect renal function of cats based on serum creatinine and urea nitrogen concentrations (*16*). However, Neerman et al. found that a subchronic dose of melamine dendrimer (40 mg/kg of body weight) led to extensive liver necrosis in mice (*17*). Previous studies in rats demonstrated that >90% of an oral dose of 0.38 mg of [^{14}C]melamine was eliminated via the kidneys in its original form within 24 h (*18*). A pharmacokinetic study of melamine in pigs suggested that melamine is readily eliminated by the kidney (*19*). When combined with other analogues such as cyanuric acid, melamine shows kidney toxicity due to crystal formation, which leads to urolith formation and renal failure (*20*−*24*).

Most of the previous studies regarding melamine toxicity have been focused on mammals. Few studies on the risk assessment of melamine in domestic animals have been reported (*19*, *25*). More information on the toxicity of melamine on domestic animals is needed because of the link between animals and public health. In this study, we investigated the toxicity of melamine in laying ducks by evaluating the biochemical and histopathological changes caused by graded dietary levels of melamine. The melamine residue in eggs was also measured to determine the extent to which melamine in duck feed can be transferred to the human food supply.

MATERIALS AND METHODS

Melamine. Melamine (purity ≥ 99.5%) was purchased from Beijing Chemical Reagent Co. (Beijing, China). Melamine was diluted with corn meal to concentrations of 1 g/kg for the formulation of high-melamine diets and to 0.1 g/kg for the formulation of low-melamine diets. These two diluted materials were then mixed in basal diets (**Table 1**), respectively, to prepare treatment diets supplemented with 1, 5, 25, 50, or 100 mg of

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Table 1. Composition and Nutrient Contents in the Basal Diet

	content
ingredient	
corn (%)	59.08
soybean meal (%)	26.60
limestone (%)	8.30
fish meal (CP, 56.2%) (%)	1.50
dicalcium phosphate (%)	1.40
wheat bran (%)	1.00
salt (%)	0.32
soybean oil (%)	0.80
vitamin[a] and mineral[b] premix	1.00
nutrient composition[c]	
metabolizable energy (ME) (MJ/kg)	11.17
crude protein (CP) (%)	17.00
calcium (%)	3.13
total phosphorus (%)	0.59
non-phytate phosphorus (%)	0.38
lysine (%)	0.91
methionine (%)	0.37
methionine + cysteine (%)	0.72

[a] Provided per kilogram of feed: vitamin A, 7800 IU; vitamin D_3, 2100 IU; vitamin K_3, 2.55 mg; vitamin E, 15 IU, vitamin B_1, 0.98 mg; vitamin B_2, 8 mg; vitamin B_6, 2.5 mg; vitamin B_{12}, 0.006 mg; calcium pantothenate, 6.00 mg; niacin, 50 mg; biotin, 0.12 mg; folic acid, 0.97 mg. [b] Provided per kilogram of feed: zinc, 100 mg; iron, 80 mg; manganese, 80 mg; copper, 16 mg; iodine, 1 mg; selenium, 0.3 mg. [c] Analyzed values except ME.

melamine/kg of diet. The basal diet was formulated to contain adequate concentrations of all nutrients required for laying ducks according to the National Research Council (NRC, 1994) (*26*). The feed samples of all treatment groups were collected to determine the actual melamine concentrations.

Birds, Feeding, and Management. This study was approved by the Animal Care and Use Committee of the Feed Research Institute of the Chinese Academy of Agricultural Sciences. Jinding laying ducks ($n = 648$, 19 weeks old), from a commercial company (Sunzhuang Duck Breeding Farm, Jingxing County, Hebei, China), with mean body weight of 1.66 ± 0.16 kg and mean egg production of 75.15 ± 3.29%, were randomly allocated to one of the six dietary treatments with six replicates each. Six experimental diets were formulated with 0, 1, 5, 25, 50, or 100 mg of melamine/kg of diet. Prior to the feeding trial, all of the birds were fed the basal diet (**Table 1**) for 14 days. After this adaptation period, the birds were initially fed the melamine-containing diets for 21 days (the treatment period) and then were fed the basal diet without melamine for 21 days (the withdrawal period). The diets were fed in mash form (water/feed, v/v = 2:1) during the entire experimental period. Ducks were maintained on a 16 h light schedule and allowed ad libitum access to experimental diets and water. Room temperature was maintained at 15 ± 2 °C.

Observation and Sample Collection. From day 1 to day 21, egg weight and egg production were recorded in replicates at 8:30 a.m. every day. Mortality was recorded daily. Feed intake was recorded daily, and feed conversion was calculated.

Five eggs were collected from each replicate on days 0, 1, 2, 3, 7, 14, and 21. At days 22, 23, 24, 25, 26, 27, 31, 34, 38, and 42, the same samples from the groups of 5, 25, 50, or 100 mg of melamine/kg of diet were collected. All of the eggs were stored at 4 °C until analysis.

On day 21, two laying ducks from each replicate were randomly selected. Body weights were recorded following an overnight fast. Blood was collected via wing vein and centrifuged at 3000*g* for 10 min to harvest serum, which was then stored at −20 °C until analysis. Ducks were immediately sacrificed by cervical dislocation. The livers and kidneys were excised and weighed. A portion of liver and kidney tissue was fixed in 10% buffered neutral formalin (Sinopharm Chemical Reagent Beijing Co., Ltd., Beijing, China), respectively.

Preparation of Standard Solutions. The stock solution of 1000 μg/mL of melamine was prepared as follows: 100 mg of melamine was transferred into a 100 mL volumetric flask and diluted with 0.2% formic acid in water. Six-point calibration standards of melamine were prepared at 1, 5, 25, 50, 100, and 250 ng/mL by diluting intermediate standard mixture solutions with acetonitrile, which contained 20 ng/mL [$^{15}N_3$]-melamine (Cambridge Isotope Laboratories, Inc., Andover, MA).

Table 2. Recovery of Melamine from Duck Eggs

fortification level (ng/g)	*n*	mean recovery (%)	RSD (%)
20	6	98.5	2.3
40	6	94.2	3.2
80	6	91.7	3.6

Egg Sample Preparation. For the determination of melamine residues, the egg samples were broken and the shell and shell membrane were discarded. The liquid of the five eggs from each replicate was homogenized at 10000 rpm for 1 min using a disperser (Ultra-Turrax model T25, IKA Werke, Staufen, Germany). Two grams of homogenized egg sample was weighed into a 15 mL screw-cap glass test tube, and 40 μL of a [$^{15}N_3$]-melamine standard solution (10 mg/L in acetonitrile) was added. The sample was vortexed for 1 min with a 10 mL mixture of acetonitrile and water (70:30, v/v) for melamine analysis.

After being sonicated in a sonic bath at 50 °C for 30 min and centrifuged at 16100*g* for 10 min, 3 mL of the supernatant was transferred to 10 mL tubes and vortexed for 1 min with 2 mL of *n*-hexane. After centrifugation at 16100*g* for 5 min, the upper organic phase (*n*-hexane) was carefully discarded, and 0.5 mL of the bottom extract was vortexed for 1 min with 0.5 mL of acetonitrile. After centrifugation at 16100*g* for 10 min, the supernatant was filtered through a 0.22 μm filter into a 2 mL autosampler vial.

Quantification of Egg Melamine Concentrations. The above egg samples were quantified for the concentrations of melamine using a LC-MS/MS (Agilent Technologies, Santa Clara, CA) method, described in detail elsewhere (*25*, *27*). To evaluate the accuracy and precision of the method, a recovery study was carried out. Untreated egg samples used for method validation were first analyzed according to the method described above, and no melamine residue was detected. A total of 5.0 g of egg samples was fortified to produce samples ($n = 6$) containing 20, 40, and 80 ng/g of melamine. Samples were kept at room temperature for at least 15 min before proceeding with extraction. These samples were analyzed by LC-MS/MS, and the signal-to-noise (S/N) ratio was recorded. The limit of detection (LOD) and limit of quantification (LOQ) for each analyte were considered to be concentrations in egg samples that produced S/N ratios of 3 and 10, respectively.

Biochemical Serum Analysis. The levels of serum urea nitrogen (BUN), creatinine (CRE), and uric acid (UC) and the activities of glutamic-pyruvic transaminase (GPT), glutamic-oxaloacetic transaminase (GOT), alkaline phosphomonoesterase (ALP), and γ-glutamyl transpeptidase (γ-GT) in serum were determined with commercial diagnostic kits (Jiancheng Bio Co., Nanjing, China) and the automatic biochemical analyzer (CE-CX5, Beckman Corp., Fullerton, CA).

Liver and Kidney Assessment. The relative kidney weight was calculated because kidney weight is relatively sensitive to nephrotoxicity. The relative weights of liver and kidney were calculated according to the following formula (*28*):

$$\text{relative organ wt (g/kg)} = \text{organ wt (g)}/\text{live body wt (kg)}$$

For histopathological studies, the formalin-fixed liver and kidney samples were stained with hematoxylin and eosin (HE) according to the method of Jadhav et al. (*29*). All reagents used were of analytical grade (Sinopharm Chemical Reagent Beijing Co., Ltd. Beijing, China). The histopathological alterations were examined with a light microscope (BX51, Olympus Corp., Tokyo, Japan).

Statistical Analyses. All data were analyzed using the one-way analysis of variance (ANOVA), and means were compared by Duncan's multiple-range test (SAS Institute, 2001). Effects were considered to be significant when $P < 0.05$. A nonlinear regression model (GraphPad Prism 4) was used to estimate the relationship between the residue of melamine in the eggs and withdrawal time.

RESULTS AND DISCUSSION

Method Performance. In our LC-MS/MS experiments, calibration curves for melamine typically gave R^2 values > 0.9958 for the egg samples. At the same time, melamine was extracted from

Table 3. Effects of Dietary Melamine Supplemental Levels on Laying Performance of Ducks Fed the Experimental Diets for 21 Days[a]

	melamine supplemental level (mg/kg of diet)					
parameter	0	1	5	25	50	100
av egg wt (g/egg)	67.5 ± 2.1	67.3 ± 1.6	67.3 ± 0.9	67.0 ± 2.0	66.8 ± 1.9	66.5 ± 1.7
egg production (%)	75.5 ± 3.0	74.2 ± 5.5	74.0 ± 4.4	74.6 ± 2.3	73.1 ± 5.0	73.6 ± 3.4
feed intake (g/duck/day)	186.5 ± 7.5	187.1 ± 17.9	185.8 ± 5.8	185.1 ± 10.6	180.5 ± 10.2	181.3 ± 16.6
feed conversion (feed/egg, g/g)	3.5 ± 0.4	3.8 ± 0.6	3.8 ± 0.5	3.8 ± 0.5	3.5 ± 0.4	3.7 ± 0.2

[a] Data are expressed as means ± SD ($n = 6$).

Table 4. Effects of Dietary Melamine Supplemental Levels on the Serum Parameters of Laying Ducks Fed the Experimental Diets for 21 Days[a]

	melamine supplemental level (mg/kg of diet)					
parameter[b]	0	1	5	25	50	100
BUN (mmol/L)	0.36 ± 0.09 b	0.38 ± 0.08 b	0.40 ± 0.07 b	0.42 ± 0.08 b	0.58 ± 0.11 a	0.62 ± 0.08 a
CRE (μmol/L)	7.8 ± 1.3	7.8 ± 0.8	8.2 ± 1.3	8.8 ± 0.8	8.8 ± 0.8	8.6 ± 1.5
UC (μmol/L)	303.0 ± 53.9	311.0 ± 44.1	327.4 ± 55.5	344.6 ± 49.5	336.2 ± 42.2	360.0 ± 25.3
GPT (U/L)	71.8 ± 16.6	71.6 ± 4.4	71.4 ± 13.9	73.8 ± 10.5	80.6 ± 16.5	76.4 ± 21.6
GOT (U/L)	64.8 ± 9.0	67.2 ± 14.9	65.8 ± 15.5	72.2 ± 13.3	71.8 ± 17.0	68.4 ± 17.0
ALP (U/L)	148.6 ± 27.0	144.2 ± 25.6	149.4 ± 23.6	145.8 ± 22.8	156.6 ± 29.5	148.8 ± 19.4
γ-GT (U/L)	13.6 ± 3.4	12.8 ± 2.3	13.6 ± 3.6	13.6 ± 1.5	13.6 ± 1.3	12.2 ± 2.3

[a] Data are expressed as means ± SD ($n = 12$). [b] BUN, blood urea nitrogen; CRE, creatinine; UC, uric acid; GPT, glutamic-pyruvic transaminase; GOT, glutamic-oxaloacetic transaminase; ALP, alkaline phosphatase; γ-GT, γ-glutamyl transpeptidase. Means with different letters within a row differ significantly ($P < 0.05$).

Table 5. Effects of Dietary Melamine on the Relative Liver and Kidney Weights of Laying Ducks Fed the Experimental Diets for 21 Days[a]

	melamine supplemental level (mg/kg of diet)					
parameter	0	1	5	25	50	100
rel liver wt (g/kg)	24.3 ± 1.0	24.7 ± 1.0	23.5 ± 1.9	25.0 ± 4.0	25.2 ± 2.7	25.5 ± 2.2
rel kidney wt (g/kg)	5.5 ± 0.4 b	5.7 ± 0.4 ab	5.9 ± 0.4 ab	5.9 ± 0.4 ab	5.9 ± 0.4 ab	6.3 ± 0.2 a

[a] Data are expressed as means ± SD ($n = 12$). Means with different letters within a row differ significantly ($P < 0.05$).

fortified samples, and recoveries of each egg sample and fortification level are presented in **Table 2**. The recoveries from fortified egg samples for melamine were 91.7−98.5% over the concentration range of 20−80 ng/g. For each fortification level, relative standard deviation (RSD) values of melamine ranged from 2.3 to 3.6%. The LOQ for melamine in samples, defined as concentration producing a S/N ratio of 10, was 20 ng/g in egg samples. The LOD for melamine, defined as the concentration that produced a S/N ratio of 3, was 10 ng/g in egg samples. The detection limit for melamine in eggs was 0.02 mg/kg.

Laying Performance. No visible signs of illness or mortality were observed during the treatment period. There were no adverse effects of graded levels of melamine in feed on the average egg weight, egg production, feed intake, and feed conversion ($P > 0.05$) (**Table 3**).

Melamine may migrate into foodstuffs from food-packaging materials (*30*, *31*). Melamine has low toxicity when administered alone (*16*, *19*, *21*). In this study, we found that diets containing different levels of melamine had no obvious adverse effects on the laying performance of ducks. Similar results were reported in broilers fed graded levels (2−1000 mg/kg of diet) of melamine in diets (*32*). However, previous studies in other animals revealed a decrease of feed intake in rats (*24*) and fish (*33*) fed diets containing 3 and 1% melamine, respectively. In the present study, the decrease of feed intake in ducks was not significant with dietary supplementation of 1−100 mg of melamine/kg of diet. Thus, it was indicated that dietary melamine as high as 100 mg/kg of diet was tolerable, but higher melamine levels may cause toxicity in ducks.

Effects of Melamine on Serum Parameters and Liver and Kidney Histology. Serum parameters were determined in laying ducks given melamine-supplemented diets for 21 days. Supplemental melamine levels ≥50 mg/kg significantly increased BUN levels in serum ($P < 0.05$) (**Table 4**). The relative kidney weight was significantly increased in the group of 100 mg of melamine/kg of feed ($P < 0.05$) (**Table 5**). The relative liver weight of ducks fed 25−100 mg of melamine/kg of feed tended to be higher ($P > 0.05$) than that of the control.

Histological lesions were observed in the kidneys of ducks subjected to ≥25 mg of melamine/kg of feed (**Figure 1**). Compared with the control group (**Figure 1A**), there were no obvious changes in the kidney structure of ducks fed 1 or 5 mg of melamine/kg of feed (**Figure 1B**,**C**). Tubular cell necrosis and lymphocytic infiltration of kidney were observed in most of the ducks fed 25 mg of melamine/kg of feed (**Figure 1D**). The kidney lesions became more severe with increasing dietary melamine dosage (**Figure 1D**−**F**). Melamine crystals were not found in the kidneys of any ducks.

Histological lesions in liver tissues from birds fed melamine-supplemented diets were less prominent than those observed in kidneys (**Figure 2**). Compared with the livers of the control group (**Figure 2A**), no obvious lesions were found in the livers of ducks fed diets supplemented with 1 or 5 mg of melamine/kg of feed (**Figure 2B**,**C**). Fatty degeneration and liver fibrosis were occasionally observed in the livers of the ducks fed diets containing 25 mg of melamine/kg of feed (**Figure 2D**). Piecemeal necrosis and inflammatory cells were present within the adjacent interstitium in some livers of the ducks fed 50 mg of melamine/kg of feed (**Figure 2E**). Liver cell vaculoation, inflammatory cell infiltration, and fatty degeneration were found in some liver of the ducks fed with 100 mg of melamine/kg of feed (**Figure 2F**).

In a clinical setting, liver or kidney injury is often detected using a battery of blood tests. We measured serum BUN, CRE, and UC levels to evaluate kidney function (*28*, *34*) and GPT, GOT, ALP,

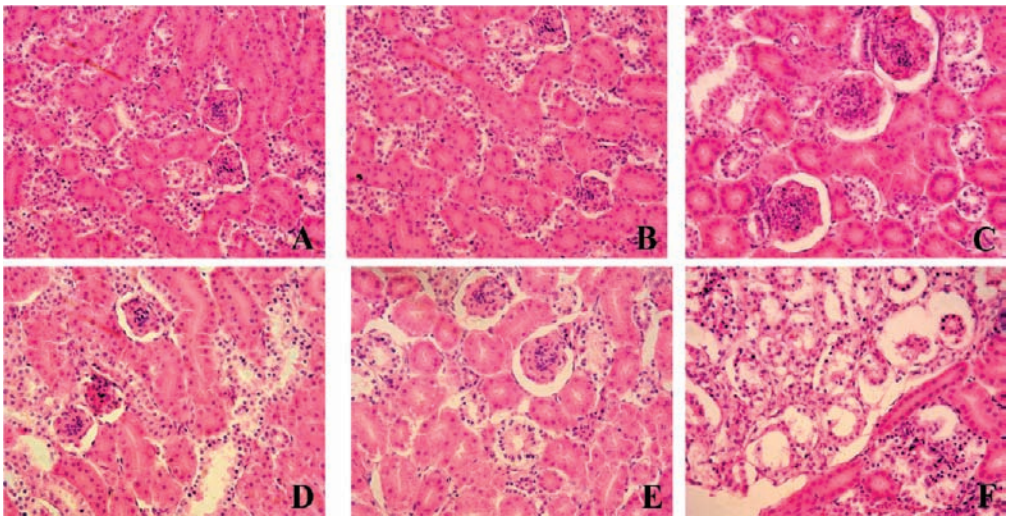

Figure 1. Effect of dietary melamine supplementation on kidney histopathology of laying ducks fed the experimental diets for 21 days (HE staining, original magnification, 40×): (**A**) control; (**B**) 1 mg of melamine/kg of diet; (**C**) 5 mg of melamine/kg of diet; (**D**) 25 mg of melamine/kg of diet; (**E**) 50 mg of melamine/kg of diet; (**F**) 100 mg of melamine/kg of diet.

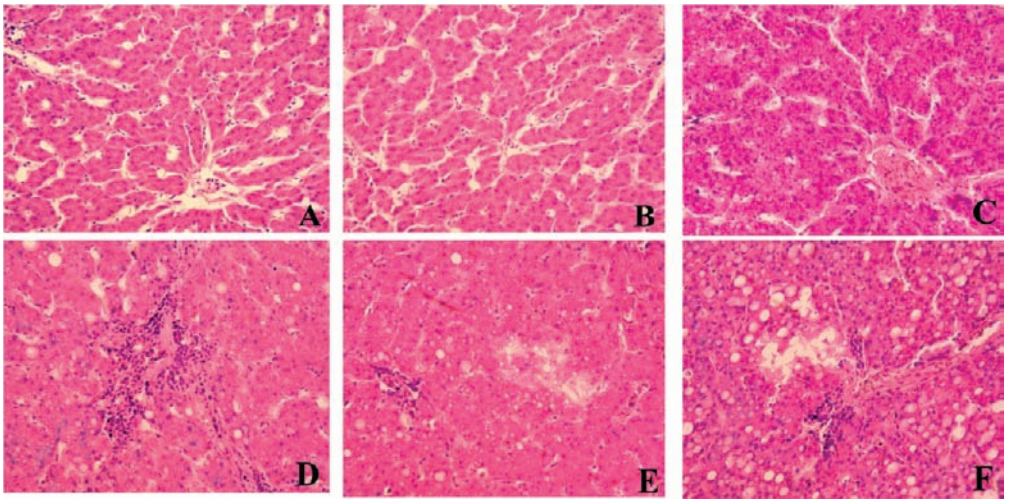

Figure 2. Effect of dietary melamine supplementation on liver histopathology of laying ducks fed the experimental diets for 21 days (HE staining, original magnification, 40×): (**A**) control; (**B**) 1 mg of melamine/kg of diet; (**C**) 5 mg of melamine/kg of diet; (**D**) 25 mg of melamine/kg of diet; (**E**) 50 mg of melamine/kg of diet; (**F**) 100 mg of melamine/kg of diet.

and r-GT levels for liver function (*35*). We also assessed the kidney and liver structure by histopathological analysis. The relative kidney weight was calculated because kidney weight is relatively sensitive to nephrotoxicity (*36*).

In the present study, dietary melamine supplementation significantly increased BUN levels. The results of relative organ weight coincided with the histopathological assessment. These were in agreement with the previous reports (*28*, *37*) that melamine increased BUN and CRE levels and led to kidney damage in cats and rats. Our results suggested that melamine can induce some histological lesions in the duck kidney. Thus, the kidney may be an important target organ for melamine toxicity.

In our study, injury in the liver of ducks was mild. Neerman observed extensive liver necrosis and increased GPT activity in mice after injection with melamine dendrimer at 40 mg/kg of body weight (*17*). Liu et al. (*33*) reported that feeding a diet containing 10000 mg of melamine/kg of diet increased ALP levels in Japanese sea bass, but the levels of GPT and GOT activity were not affected. One possible reason for this different response of liver function may be that the melamine supplemental levels were not sufficient to induce obvious injury to the liver in our study. Second, the toxicity is likely to differ between melamine and melamine dendrimer, as different chemical structures may contribute to different characteristics. In addition, the toxicity of melamine may differ in various animal species.

The formation of melamine cyanurate may be related to the UC concentration and the pH of urine, which may cause precipitation in the tubules (*28*). In our study, the serum UC concentrations were slightly increased by dietary melamine supplementation, but no crystallization was found in the duck kidney. This may be partly explained by UC concentration lower than the threshold necessary for crystallization. Moreover, much lower melamine levels (≤100 mg/kg of diet) were applied in the present study than in other studies (≥10000 mg/kg of diet) (*23*, *24*).

Melamine Accumulation in Eggs. From day 1 to day 21, melamine residue in the eggs of ducks fed 1 and 5 mg of melamine/kg of diet slightly increased (**Figure 3**). However, there were no significant differences in egg residue among the groups of 0, 1, and 5 mg of melamine/kg of diet ($P > 0.05$). When the ducks

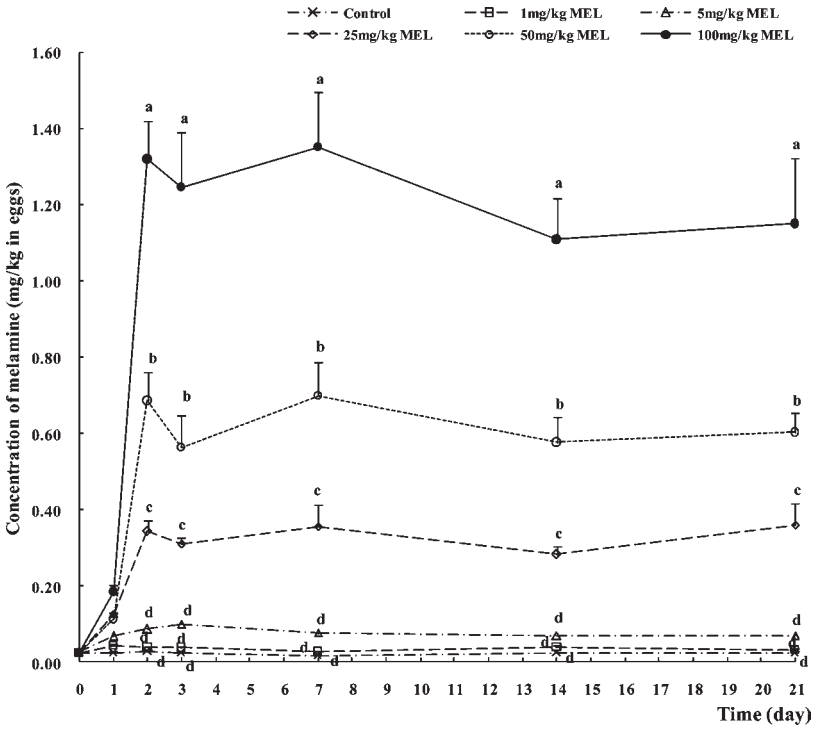

Figure 3. Effects of dietary melamine supplemental levels on the melamine concentrations in eggs of laying ducks fed the experimental diets for 21 days (mg/kg, fresh weight basis). Means with different letters within a day differ significantly ($P < 0.05$). Error bars represent standard deviations; where not seen, they lie within the symbol ($n = 6$).

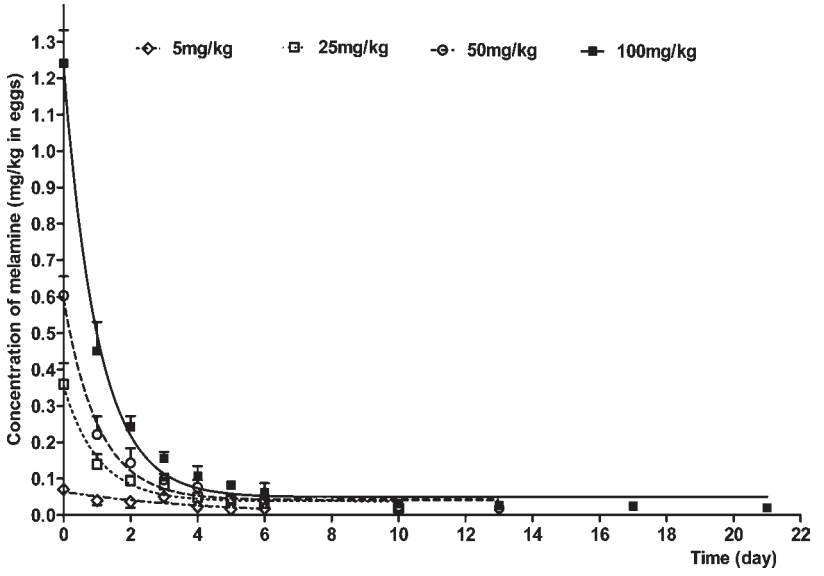

Figure 4. Melamine concentration changes in the eggs of laying ducks during the withdrawal period. *Y* is the concentration of melamine in the eggs of laying ducks (mg/kg); *X* is the depletion time (days). Error bars represent standard deviations; where not seen, they lie within the symbol ($n = 6$).

were given diets containing higher levels of melamine (25−100 mg/kg of feed), melamine residue in the eggs was significantly increased ($P < 0.05$) (**Figure 3**). Melamine concentrations in the eggs from groups of 25−100 mg of melamine/kg of diet reached a high level 2 days after the melamine-supplemented diet was supplied and then fluctuated continually around high levels until day 21.

Melamine is primarily eliminated by renal filtration in rats (*18*) and pigs (*19*) and does not undergo significant metabolism in many animals (*38*). In this study, melamine levels in the eggs of laying ducks increased with dietary melamine levels. Lü et al. (*32*) reported that melamine residue in broiler tissues increased with dietary melamine levels. The results of laying ducks suggested that dietary melamine can be transferred to eggs. The concentration of melamine in eggs increased as the melamine supplemental level increased.

Melamine Depletion in Eggs. To investigate the depletion mode of melamine residue in eggs, the melamine concentrations in the eggs of the ducks fed higher levels of melamine (5−100 mg/kg of diet) were examined during the withdrawal period. The results showed that melamine concentration in the eggs of laying ducks decreased rapidly after melamine was removed from the feed of the laying ducks (**Figure 4**). The half-life ($t_{1/2}$) of melamine in the eggs of laying ducks was determined by nonlinear regression

analysis (GraphPad Prism 4) based on the melamine concentrations in eggs during the withdrawal period, and the model was

$$Y = (Y_0 - \mathrm{NS}) \times \exp(-K \times X) + \mathrm{NS}$$

where Y is the concentration of melamine in the eggs, mg/kg; X is the depletion time, day; Y_0 is the binding at time zero; NS is the binding (nonspecific) at infinite times; and K is the rate constant. $t_{1/2} = \ln 2/K$.

The depletion equations for 5, 25, 50, and 100 mg/kg groups were as follows: $Y = 0.0620 \times \exp(-0.2407X) + 0.0026$ ($R^2 = 0.7744$, $P = 0.9666$); $Y = 0.3171 \times \exp(-0.9685X) + 0.0384$ ($R^2 = 0.9805$, $P = 0.0226$); $Y = 0.5541 \times \exp(-0.9658X) + 0.0417$ ($R^2 = 0.9871$, $P = 0.0160$); $Y = 1.1803 \times \exp(-0.9714X) + 0.0497$ ($R^2 = 0.9935$, $P = 0.0056$), respectively. The correlation coefficient of the equations for the group of 5 mg/kg was much lower compared to those for other groups. This may be caused by analysis error because of too low melamine levels, close to the detection limit (0.02 mg/kg), in the eggs. On the basis of the equations of the groups of 25, 50, and 100 mg of melamine/kg of feed (**Figure 4**), the half-lives of melamine in the eggs were 0.72, 0.72, and 0.71 days, respectively. Egg melamine rapidly reached the $t_{1/2}$ time point (< 18 h), suggesting that ducks clear this chemical efficiently and that excessive melamine has little opportunity to accumulate in the animal product (*19*).

According to USDA, the current safety level of melamine in meat products should be < 0.05 mg/kg (*39*). On the basis of these depletion equations and the melamine safety level, the periods required for melamine residue in the eggs of ducks fed 25, 50, and 100 mg of melamine/kg of diet to decrease to ≤ 0.05 mg/kg in eggs were 3.41, 4.34, and 8.52 days, respectively. Thus, the period required for melamine depletion was longer for ducks that received a higher level of dietary melamine. Similar findings have reported that 20 and 21.3 h were required for depletion of melamine in the kidney of pigs after withdrawal of oral doses of 3.0 and 5.12 mg of melamine/kg of feed, respectively (*40*).

On the basis of the above results, we assumed that most of the melamine within the animal body existed in a free form, which allowed for rapid depletion at the beginning of the withdrawal period. Higher concentrations of melamine may be widely distributed in different tissues, requiring more time to be cleared.

ABBREVIATIONS USED

ANOVA, analysis of variance; ALP, alkaline phosphomonoesterase; BUN, urea nitrogen; CP, crude protein; CRE, creatinine; GOT, glutamic-oxaloacetic transaminase; GPT, glutamic-pyruvic transaminase; HE, hematoxylin and eosin; HILIC, hydrophilic interaction liquid chromatography; LC-MS/MS, liquid chromatography−tandem mass spectrometry; ME, metabolizable energy; MJ, megajoule; γ-GT, γ-glutamyl transpeptidase; SAS, Statistical Analysis System; SD, standard deviation; $t_{1/2}$, half-life; UC, uric acid; USDA, U.S. Department of Agriculture.

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LITERATURE CITED

(1) Anderson, F. A. Final report on the safety assessment of melamine/formaldehyde resin. *J. Am. Coll. Toxicol.* **1995**, *14*, 373–385.

(2) Subrayan, R. P.; Rasmussen, P. G. An overview of materials composed of carbon and nitrogen. *Trends Polym. Sci.* **1995**, *3*, 165–172.

(3) Weil, E. D.; Choudhary, V. Flame-retarding plastics and elastomers with melamine. *J. Fire Sci.* **1995**, *13*, 104–126.

(4) Lim, L. O.; Scherer, S. J.; Shuler, K. D. Disposition of cyromazine in plants under environmental conditions. *J. Agric. Food Chem.* **1990**, *38*, 860–864.

(5) Cabras, P.; Meloni, M.; Spanedda, L. High performance liquid chromatographic separation of cyromazine and its metabolite melamine. *J. Chromatogr.* **1990**, *505*, 413–416.

(6) Shelton, D. R.; Karns, J. S.; McCarty, G. W. Metabolism of melamine by *Klebsiella terragena*. *Appl. Environ. Microbiol.* **1997**, *63*, 2832–2835.

(7) Newton, G. L.; Utley, P. R. Melamine as a dietary nitrogen source for ruminants. *J. Anim. Sci.* **1978**, *25*, 126–128.

(8) Thomas, R.; Kulkarni, G. U. A hydrogen-bonded channel structure formed by a complex of uracil and melamine. *Beilstein J. Org. Chem.* **2007**, *28*, 3–17.

(9) Burns, K. Recall shines spotlight on pet foods. *J. Am. Vet. Med. Assoc.* **2007**, *230*, 1285–1288.

(10) Lachenmeier, D. W.; Humpfer, E.; Fang, F.; Schütz, B.; Dvortsak, P.; Sproll, C.; Spraul, M. NMR-spectroscopy for nontargeted screening and simultaneous quantification of health-relevant compounds in foods: the example of melamine. *J. Agric. Food Chem.* **2009**, *57*, 7194–7199.

(11) Burns, K. Events leading to the major recall of pet foods. *J. Am. Vet. Med. Assoc.* **2007**, *230*, 1601–1604.

(12) Nestle, M.; Nesheim, M. C. Additional information on melamine in pet food. *J. Am. Vet. Med. Assoc.* **2007**, *231*, 1647.

(13) Ingelfinger, J. R. Melamine and the global implications contamination. *N. Engl. J. Med.* **2008**, *359*, 2745–2748.

(14) Guan, N.; Fan, Q.; Ding, J.; Zhao, Y.; Lu, J.; Ai, Y.; Xu, G.; Zhu, S.; Yao, C.; Jiang, L.; Miao, J.; Zhang, H.; Zhao, D.; Liu, X.; Yao, Y. Melamine-contaminated powdered formula and urolithiasis in young children. *N. Engl. J. Med.* **2009**, *360*, 1067–1074.

(15) The Government of the Hong Kong Special Administrative Region Centre for Food Safety. Latest test results for melamine (with table), Oct 28, 2008; http://www.cfs.gov.hk/english/press/2008_10_25_1_e.html.

(16) Puschner, B.; Poppenga, R. H.; Lowenstine, L. J.; Filigenzi, M. S.; Pesavento, P. A. Assessment of melamine and cyanuric acid toxicity in cats. *J. Vet. Diagn. Invest.* **2007**, *19*, 616–624.

(17) Neerman, M. F.; Zhang, W.; Parrish, A. R.; Simanek, E. E. *In vitro* and *in vivo* evaluation of a melamine dendrimer as a vehicle for drug delivery. *Int. J. Pharm.* **2004**, *281*, 129–132.

(18) Mast, R. W.; Jeffcoat, A. R.; Sadler, B. M.; Kraska, R. C.; Friedman, M. A. Metabolism, disposition and excretion of melamine in male Fischer 344 rats. *Food Chem. Toxicol.* **1983**, *21*, 807–810.

(19) Baynes, R. E.; Smith, G.; Mason, S. E. Pharmacokinetics of melamine in pigs following intravenous administration. *Food Chem. Toxicol.* **2008**, *46*, 1196–1200.

(20) Melnick, R. L.; Borman, G. A.; Haseman, J. K. Urolithiasis and bladder carcinogenicity of melamine in rodents. *Toxicol. Appl. Pharmacol.* **1984**, *72*, 292–303.

(21) Reimschuessel, R.; Gieseker, C. M.; Miller, R. A.; Ward, J.; Boehmer, J.; Rummel, N.; Heller, D. N.; Nochetto, C.; de Alwis, G. K.; Bataller, N.; Andersen, W. C.; Turnipseed, S. B.; Karbiwnyk, C. M.; Satzger, R. D.; Crowe, J. B.; Wilber, N. R.; Reinhard, M. K.; Roberts, J. F.; Witkowski, M. R. Evaluation of the renal effects of experimental feeding of melamine and cyanuric acid to fish and pigs. *Am. J. Vet. Res.* **2008**, *69*, 1217–1228.

(22) Brown, C. A.; Jeong, K. S.; Poppenga, R. H.; Puschner, B.; Miller, D. M.; Ellis, A. E.; Kang, K.; Sum, S.; Cistola, A. M.; Brown, S. A. Outbreaks of renal failure associated with melamine and cyanuric acid in dogs and cats in 2004 and 2007. *J. Vet. Diagn. Invest.* **2007**, *19*, 525–531.

(23) Okumura, M.; Hasegawa, R.; Shirai, T.; Ito, M.; Yamada, S.; Fukushima, S. Relationship between calculus formation and carcinogenesis in the urinary bladder of rats administered the

non-genotoxic agents thymine or melamine. *Carcinogenesis* **1992**, *13*, 1043–1045.
(24) Ogasawara, H.; Imaida, K.; Ishiwata, H.; Toyoda, K.; Kawanishi, T.; Uneyama, C.; Hayashi, S.; Takahashi, M.; Hayashi, Y. Urinary bladder carcinogenesis induced by melamine in F344 male rats: correlation between carcinogenicity and urolith formation. *Carcinogenesis* **1995**, *16*, 2773–2777.
(25) Lv, X. W.; Wang, J.; Wu, L.; Qiu, J.; Li, J. G.; Wu, Z. L.; Qin, Y. C. Tissue deposition and residue depletion in lambs exposed to melamine and cyanuric acid-contaminated diets. *J. Agric. Food Chem.* **2010**, *58*, 943–948.
(26) National Research Council. *Nutrient Requirements of Poultry*, 9th revised ed.; National Academy Press: Washington, DC, 1994; pp 42−43.
(27) NY-PRC, Codex standard for simultaneous determination of melamine and cyanuric acid in animal blood by liquid chromatography−triple quadrupole mass spectrometry, **2009**.
(28) 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.; Reimschussel, R.; Overmann, G.; Shan, Y.; Daston, G. P. Identification and characterization of toxicity of contaminants in pet food leading to an outbreak of renal toxicity in cats and dogs. *Toxicol. Sci.* **2008**, *106*, 251–262.
(29) Jadhav, S. H.; Sarkar, S. N.; Patil, R. D.; Tripathi, H. C. Effects of subchronic exposure via drinking water to a mixture of eight water-contaminating metals: a biochemical and histopathological study in male rats. *Arch. Environ. Contam. Toxicol.* **2007**, *53*, 667–677.
(30) Bradley, E. L.; Boughtflower, V.; Smith, T. L.; Speck, D. R.; Castle, L. Survey of the migration of melamine and formaldehyde from melamine food contact articles available on the UK market. *Food Addit. Contam.* **2005**, *22*, 597–606.
(31) Lu, J.; Xiao, J.; Yang, D. J.; Wang, Z. T.; Jiang, D. G.; Fang, C. R; Yang, J. Study on migration of melamine from food packaging materials on markets. *Biomed. Environ. Sci.* **2009**, *22*, 104–108.
(32) Lü, M. B.; Yan, L.; Guo, J. Y.; Li, Y.; Li, G. P.; Ravindran, V. Melamine residues in tissues of broilers fed diets containing graded levels of melamine. *Poult. Sci.* **2009**, *88*, 2167–2170.
(33) Liu, H. Y.; Zhang, W.; Xue, M.; Wu, X. F.; Zheng, Y. H.; Guo, L. Y.; Sheng, H. J. Acute toxicity study for melamine on Japanese sea bass (*Lateolabrax japonicus*). *Acta Hydrobiol. Sin.* **2009**, *33*, 157−163 (in Chinese).
(34) Travlos, G. S.; Morris, R. W.; Elwell, M. R.; Duke, A.; Rosenblum, S.; Thompson, M. B. Frequency and relationships of clinical chemistry and liver and kidney histopathology findings in 13-week toxicity studies in rats. *Toxicology* **1996**, *107*, 17–29.
(35) Piñeiro-Carrero, V. M.; Piñeiro, E. O. Liver. *Pediatrics* **2004**, *113*, 1097–1106.
(36) Kluwe, W. M. Renal function tests as indicators of kidney injury in subacute toxicity studies. *Toxicol. Appl. Pharmacol.* **1981**, *57*, 414–424.
(37) Lin, X. H.; Wang, J. F.; Jia, G. L.; Mei, L.; Wang, Z. Study on the toxicity of melamine. *J. Toxicol.* **2008**, *22*, 216−218 (in Chinese).
(38) Seffernick, J. L.; Dodge, A. G.; Sadowsky, M. J.; Bumpus, J. A.; Wackett, L. P. Bacterial ammeline metabolism via guanine deaminase. *J. Bacteriol.* **2010**, *192*, 1106–1112.
(39) USDA. *Disposition of Hogs and Chickens from Farms Identified as Having Received Pet Food Scraps Contaminated with Melamine and Melamine-Related Compounds and Offered for Slaughter*; U.S. Department of Agriculture: Washington, DC, **2007**.
(40) Buur, J. L.; Baynes, R. E.; Riviere, J. E. Estimating meat withdrawal times in pigs exposed to melamine contaminated feed using a physiologically based pharmacokinetic model. *Regul. Toxicol. Pharmacol.* **2008**, *51*, 324–331.

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