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ARTICLE

Tissue Deposition and Residue Depletion in Laying Hens Exposed to Melamine-Contaminated Diets

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To investigate the deposition and elimination of melamine in hen eggs and tissues, 72 Roman laying hens were administrated with melamine at 8.6−140.9 mg per kilogram of body weight per day for 34 days. The crystals were found in one of three kidneys of hens treated with melamine at either 62.6 or 140.9 mg/kg. Furthermore, the melamine concentrations in egg, muscle, liver, kidney, stomach, duodenum, uterus, ovary, and blood plasma were determined by high-performance liquid chromatography−ultraviolet (HPLC−UV) methods. A higher dosage of melamine in the diet corresponded to higher concentrations in tissues and eggs. The concentrations of melamine in tissues were in the following ranges (μg/g): egg, 1.1−28.7; muscle, 0.4−9.3; liver, 0.5−6.9; kidney, 1.3−21.7; stomach, 0.4−7.3; duodenum, 0.3−2.8; uterus, 0.5−6.9; ovary, 0.5−9.1; and blood plasma, 0.8−7.6. When melamine was withdrawn from the diet of hens, the melamine concentration in hen tissues fell to below 2.5 μg/g by day 10 and no residues were detected in eggs or tissues at days 7 and 20, respectively.

KEYWORDS: Melamine; laying hen; deposition; elimination

INTRODUCTION

Melamine (MEL), also known as trimeric cyanamide, is a nitrogen-containing, heterocyclic triazine compound. MEL has several industrial uses, such as the manufacturing of plastics, MEL−formaldehyde resins, and flame-resistant materials. MEL contains approximately 66.6% nitrogen, and the addition of 1% MEL to proteins leads to an increase in the Kjeldahl protein content of 4.16%. Because of this nitrogen enrichment, the use of MEL as a non-protein nitrogen (N) source for cattle was first described in a patent (*1*). In 1978, however, a study concluded that MEL was not an acceptable non-protein N source for ruminants because in cattle its hydrolysis is slower and less complete than other nitrogen sources, such as urea (*2*). Previous studies have also proven that MEL was nearly unmetabolically active in rats because 90% of the MEL administered was discharged within 24 h through the kidneys (*3*−*5*). Moreover, another study showed MEL-induced crystalluria and mortality in sheep (*6*), and MacKenzie also reported weight loss and mortality in sheep receiving MEL (*7*).

Recently, MEL has become known as one of the most effective adulterants used to increase the nitrogen content in foods and feeds (*8*). In 2007, MEL contamination in imported pet food was found to be responsible for thousands of pet deaths in the U.S.A. and an incident of MEL contamination in infant formula led to serious illness in thousands of babies in China (*9*−*12*). In 2008, egg powders were found to contain MEL at concentrations ranging from 0.1 to 4.0 μg/g and fresh eggs were found to contain MEL at concentrations ranging from 2.9 to 4.7 μg/g in China (*13*). Positive results for the presence of MEL were also found in dried whole eggs in Japan at concentrations ranging from 2.8 to 4.6 μg/g, as well as in egg products from the U.S.A. at 1.1 μg/g (*14*).

Analytical platforms used for analyzing trace MEL have comprised gas chromatography coupled to mass spectrometry (GC−MS) (*15*), liquid chromatography coupled to mass spectrometry (LC−MS) (*16*), capillary electrophoresis coupled to mass spectrometry (CE−MS), gas chromatography−tandem mass spectrometry (GC−MS/MS), liquid chromatography−tandem mass spectrometry (LC−MS/MS) (*17*−*20*), and surface desorption mass spectrometry assays (*21*, *22*). Furthermore, nuclear magnetic resonance (NMR)-based methods (*8*), enzyme-linked immunosorbent assay (ELISA) (*23*), and neutral impact radiation spectroscopy (NIRS)-based methods (*24*) have also been explored as potential screening methods for the identification of contaminated products.

Because MEL was purposefully added to food or animal feed to increase the nitrogen content, it is thought that the presence of MEL in food of animal origin may be a result of carry-over from adulterated animal feed. In addition, many groups have reported that MEL combined with cyanuric acid can form MEL cyanurate

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Table 1. Ingredients and Chemical Composition of the Basal Diet (g/kg)

ingredients	
corn	66.38
soybean meal [440 g/kg of ceruloplasmin (CP)]	18.92
rapeseed meal	4.00
calcium carbonate	8.12
dicalcium phosphate	1.20
lysine	0.05
methionine	0.08
choline	0.10
sodium chloride	0.40
lucantin CX forteb	0.02
vitamin premix[a]	0.03
mineral premix[b]	0.50
bentonite[c]	0.20
calculated analysis	
crude protein (%)	15.05
metabolizable energy (Mcal)	2.65
calcium (%)	3.50
available phosphorus (%)	0.32
methionine (%)	0.32
threonine (%)	0.61
lysine (%)	0.71

[a] There are 25000 IU of V_A, 35000 IU of V_D, 12.5 IU of V_E, 32.5 mg of V_K, 1.0 mg of V_{B1}, 8 mg of V_{B2}, 3.0 mg of V_{B6}, 15 μg of V_{B12}, 250 μg of acdium, 17.5 mg of nicacid, and 12.5 mg of calcium pantothenate in every kilogram of diet. [b] There are 60 mg of Fe ($FeSO_4 \cdot 7H_2O$), 8 mg of Cu ($CuSO_4 \cdot 5H_2O$), 60 mg of Mn ($MnSO_4 \cdot H_2O$), 80 mg of Zn ($ZnSO_4 \cdot 7H_2O$), 0.3 mg of Se (Na_2SeO_3 content, 1.0% Se), and 0.35 mg of I (KI content, 3.8% I) in every kilogram of diet. [c] Bentonite should be replaced with appropriate MEL in the MEL-fortified diets.

in pigs, fish, cats, and rats. MEL cyanurate is insoluble and can crystallize in the kidneys, thus causing renal failure (*25*−*28*). However, few farm animal feeding studies are available to allow for a quantitative assessment of the possible carry-over of MEL into eggs and hen tissues from their diets. In this study, we focused on exploring the deposition of MEL in eggs and tissues of hens fed with MEL-contaminated diets. We also examined the elimination of MEL in the eggs and tissues of hens.

MATERIALS AND METHODS

Chemicals. MEL, with a purity of 99.4%, was obtained from Sigma-Aldrich (Shanghai, China). All other chemicals and solvents used in the analyses were reagent-grade.

Animals and Diet Formulation. A total of 72 Lohmann pink laying hens (55 weeks old) were randomly divided into six groups. Each group consisted of 12 hens, kept in six cages (two birds in each cage). The birds were fed *ad libitum*. The control group (I) was fed with the standard feed (**Table 1**). The second (II), third (III), fourth (IV), fifth (V), and sixth (VI) groups were given the same feed supplemented with MEL at levels of 8.6, 17.4, 33.6, 62.6, and 140.9 mg/kg of body weight (bw) per day, respectively. In this experiment, no MEL was detected in the feed materials, including corn, soybean meal, rapeseed meal, and other raw materials. The hens received 15 h of light/day throughout the experiment. The room temperature was controlled at 21 °C. The hens were fed with the same diet for up to 34 days.

Sample Collection. To evaluate the deposition of MEL in eggs, six egg samples were collected from each group at days 1−7, 10, 14, 21, 28, and 34 after administration of MEL. The eggs samples were weighed and kept at 4 °C until further analyzed.

To evaluate the clearance of MEL from eggs and tissues, all hens were switched to the control treatment after 34 days. Four egg samples were randomly collected from each group on days 1−5 and 7 following the diet switch. In addition, three hens from each group were killed with perfusion at 6 h after withdrawal of the diet on the last day of administration of MEL and 10 and 20 days after the withdrawal of MEL. Hens were first anesthetized with 20% chloral hydrate in water. The carotid arteries were then exposed in the ventral neck region and cannulated. Hens were perfused with physiological saline solution for 25 min. All tissues were then obtained from previously perfused hens, except blood. The blood, liver, kidney, muscle, masticatory stomachs, uterus, ovary, and duodenum samples were kept at −20 °C until analyzed. Furthermore, when three hens of each group were euthanized at 6 h after withdrawal of the diet on the last day of administration of MEL, samples of kidney tissues were obtained for microscopic and histologic examination. The above study protocol was approved by the Department of Animal Husbandry, Ministry of Agriculture of the People's Republic of China.

Histologic Examination of Kidney Tissue. Wet-mount sections of kidney (2 × 3 mm slices compressed between two glass slides) were immediately evaluated via light microscopy for the presence of crystals (*27*). Sections of kidney tissue were stored at −20 °C and fixed in neutral-buffered 10% formalin. Kidney tissue was processed for routine histologic evaluation, and sections (5 μm) were stained with hematoxylin and eosin.

Sample Preparation for High-Performance Liquid Chromatography (HPLC) Analysis. The egg and tissue samples were prepared for HPLC analysis using the following steps. The sample was first homogenized with a tissue homogenizer. Then, 5 g of the egg sample and 2 g of the tissue sample were weighed into a 50 mL screw-cap glass test tube, and 20 mL of diethyl ether was added for fat removal. The sample was then vortexed for 1 min with 18 mL of trichloroacetic acid and 2 mL of lead acetate. After sonication in a water bath at 50 °C for 20 min, the sample was centrifuged at 15000*g* for 10 min. The mixed-type cation-exchange column (60 mg/3 mL, Waters, Inc., Milford, MA) was activated with 3 mL of methanol and 3 mL of water, and 9 mL of the centrifuged solution was transferred to the column. The mixed-type cation-exchange column was rinsed with 3 mL of water and 3 mL of methanol and then washed with 6 mL of mixed ammonia and methanol mixed (5:100, v/v). The eluent was dried by 50 °C nitrogen. A volume of 1 mL of a 20% methanol solution (in deionized water, v/v) was added for dilution. It was mixed for 1 min in a vortex mixer and passed through a 0.45 μm filter membrane.

Quantification of MEL Concentrations. The above samples were quantified for MEL concentration using a HPLC-based method. MEL concentrations were confirmed by a GC−MS-based method (*29*) when the MEL concentration in the sample was lower than the limit of quantification (LOQ) for the HPLC-based method. The HPLC system (Agilent 1100, Böblingen, Germany) consisted of a quaternary pump, an autosampler, a degasser, an automatic thermostatic column compartment, and a computer with Chemstation software (Analyst 1.4, Applied Biosystems, Inc., Foster City, CA). The analytical column was an Agilent Zorbax 300 SB-C18 column (5 μm, 150 × 4.6 mm).

The mobile phase used was acetonitrile and water containing 10 mmol/L citric acid and 10 mmol/L sodium octane sulfonate (10:90, v/v). The mobile phase was degassed automatically using an electronic degasser system. With a flow rate of 1 mL/min at ambient temperature, the MEL presence was monitored at 240 nm, which was detected by a variable wavelength detector.

Data from the MEL analysis were subjected to analysis of variation (ANOVA) using the features of SPSS 13.0 software.

Method Validation. Untreated egg and hen tissues used for method validation were first analyzed by the method described above, and no MEL residue was detected. A total of 5.0 g of egg samples or 2.0 g portions of tissue homogenate were fortified to produce samples ($n = 5$) containing 0.2, 0.5, and 20 μg/g of MEL. Samples were kept at room temperature for at least 15 min before extraction. Samples were analyzed by HPLC−ultraviolet (UV), and the signal-to-noise (S/N) ratio was recorded. The limit of detection (LOD) and LOQ for MEL were considered to be concentrations in tissues or plasma samples that produced a S/N ratio of at least 3 and 10, respectively. Moreover, quantification limits for the HPLC−UV method were validated by GC−MS methods.

RESULTS AND DISCUSSION

Method Performance. The laying hens were fed MEL at concentrations ranging from 125 to 2000 mg/kg (approximately 8.6−140.9 mg/kg of bw), and the quantification limit for MEL in HPLC−UV analysis was 0.05 μg/g (*14*), which was a satisfaction for the analysis in our study. Although it is a lack of specificity of the HPLC-based methods, the HPLC peak profiles of the MEL standard (**Figure 1B**) and MEL from the egg sample (**Figure 1C**) or other tissues (data not shown) are consistent. In our HPLC−UV

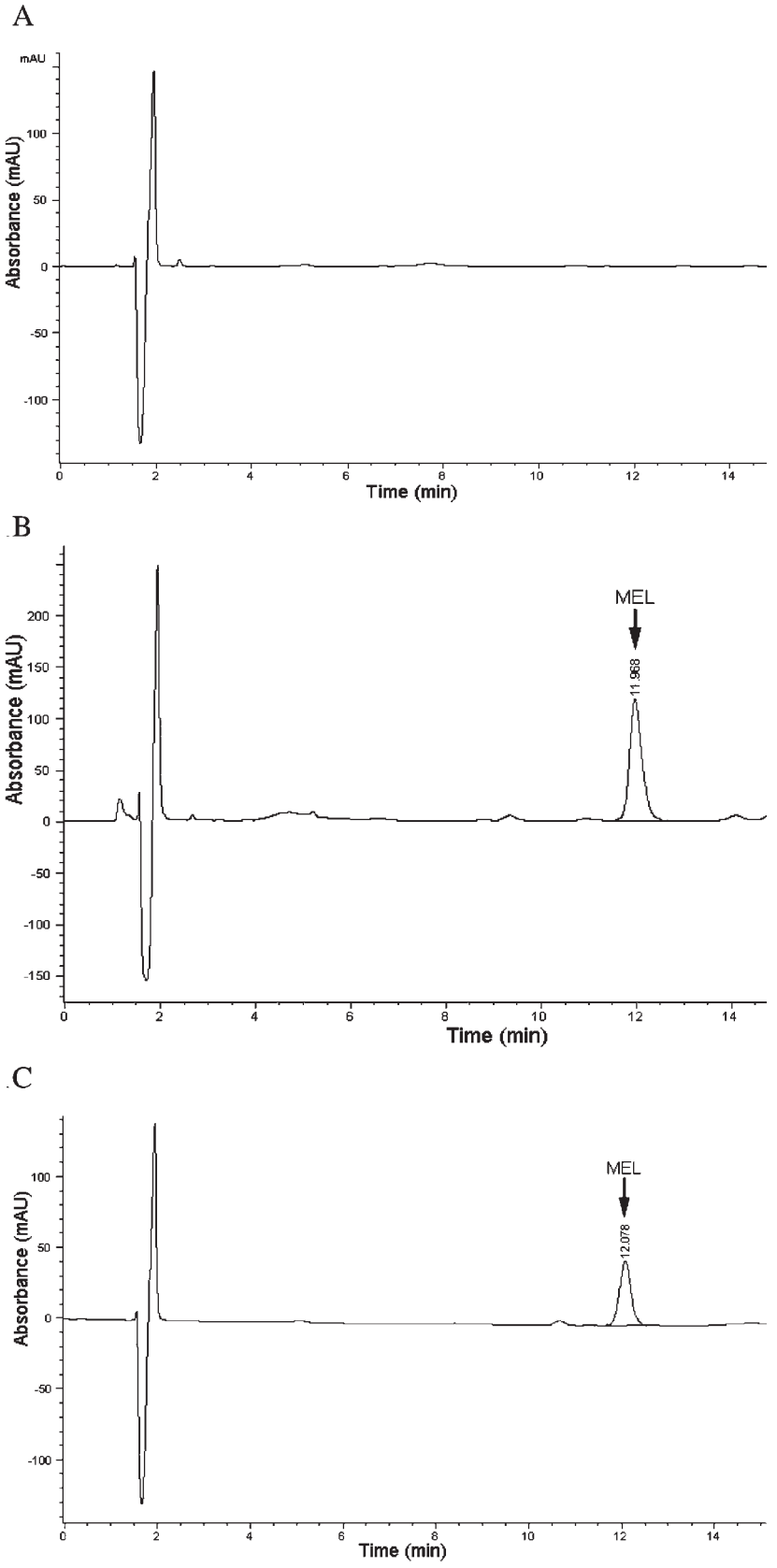

Figure 1. HPLC chromatogram relative to the (A) blank matrix, (B) MEL standard at a concentration of 5 μg/mL, and (C) MEL from the egg sample at a concentration of 3.48 μg/g.

assay, five point calibration standards of MEL were prepared at 0.5, 2, 10, 25, and 50 μg/mL and the calibration curve for MEL typically gave R^2 values of 0.9989. At the same time, MEL was extracted from the fortified samples, and recovery levels of egg are presented in **Table 2**. The revovery from fortified solid tissues or egg was 77.0−84.3% over the concentration range of 0.2−20 μg/g. The recovery from fortified plasma was 85.8−92.0% over the concentration range of 0.2−20 μg/mL. For each calibration point,

Table 2. Recovery of MEL from Fortified Tissues ($n = 5$)

sample	fortification level[a]	mean recovery (%)	RSD (%)
egg	0.2	82.5	3.5
	0.5	83.0	3.6
	20	83.3	2.9
plasma	0.2	92.0	6.1
	0.5	85.8	2.6
	20	87.0	4.2
muscle	0.2	84.3	3.8
	0.5	81.0	4.8
	20	84.0	4.3
liver	0.2	79.5	4.9
	0.5	80.3	5.0
	20	83.2	3.9
kidney	0.2	81.1	2.9
	0.5	82.4	3.7
	20	84.1	4.3
duodenum	0.2	77.0	4.9
	0.5	81.6	2.6
	20	83.9	3.2
uterus	0.2	81.9	3.3
	0.5	80.8	5.1
	20	82.5	2.7
ovary	0.2	80.1	2.6
	0.5	81.2	4.5
	20	82.8	4.0

[a] The unit of MEL concentration in solid tissues or egg is μg/g, and the unit of MEL concentration in plasma is μg/mL.

relative standard deviation (RSD) values ranged from 2.6 to 6.1%. The LOQ for MEL in samples, defined as concentrations that produced a S/N ratio of at least 10, was 0.5 μg/mL in plasma and 0.5 μg/g in solid tissues or egg. The LOD for MEL, defined as concentrations that produced a S/N ratio of 3, was 0.2 μg/mL in plasma and 0.2 μg/g in solid tissues or egg. Furthermore, in our GC−MS experiment, calibration curves for MEL gave R^2 values >0.992 for tissue and egg samples and the recoveries from fortified hen tissues or egg for MEL were 89.2−99.5% over the concentration range of 100−500 ng/g.

Clinical and Histologic Evaluation of Kidney Samples. There were no effects on the survival, body weight gain, or egg production of hens fed with 8.6−140.9 mg/kg of bw MEL for 34 days. No pathological changes were observed in the kidney of hens administered with 8.6 mg/kg of bw MEL (**Figure 2B**). However, histopathology revealed dilated renal tubules and small blood vessel expansion in hens that received MEL at 17.4−140.9 mg/kg of bw for 34 days (panels **C**−**F** of **Figure 2**). Furthermore, crystals were found in one of three kidneys of the MEL-treated hens from either group V or VI. In one recent study, Reimschuessel et al. described that chickens fed only MEL could develop spherulite crystals containing uric acid, a normal excretion product of chickens, and MEL in his unpublished data (*30*). Furthermore, previous studies have shown that pigs, fish, cats, and rats fed MEL and CYA in a 1:1 ratio develop renal crystals composed of MEL−cyanurate (*25*−*27*). However, no changes in renal functions were observed in cats and fish treated with either MEL or CYA alone at doses of 181 or 400 mg/kg of bw (*27*).

Deposition of MEL in Eggs. MEL residue was observed in eggs collected after day 2 of the diet, and the concentration of MEL peaked on day 2 when hens were administrated MEL ranging from 33.6 to 140.9 mg/kg of bw per day (**Table 3**). Preliminary data also showed that peak MEL concentrations were 12.7 μg/g in catfish and 12.2 μg/g in trout on day 1 following administration of MEL at 20 mg/kg of bw (*17*). In rats, MEL was also rapidly absorbed from the intestine and attained maximal plasma concentrations in 1 h following a single oral dose (*3*).

MEL residue concentrations in eggs increased following greater amounts of MEL in the diet. MEL concentrations in eggs from groups IV, V, and VI were significantly higher than those from groups II and III, but there was no difference in MEL concentrations in eggs from groups II and III. However, there was a tendency for MEL concentrations to increase as the treatment time increased in groups II and III, and the MEL concentrations in eggs stabilized at days 4 and 3 for groups II and III, respectively. There was no change in MEL concentrations in eggs from groups IV, V, and VI. At last, peak MEL concentrations were 1.6, 3.0, 6.7, 11.7, and 28.7 μg/g in egg samples with administration of 8.6, 17.4, 33.6, 62.6, and 140.9 mg/kg of bw per day within 34 days, respectively.

Deposition of MEL in Plasma and Tissues of Laying Hens. MEL residues in plasma and tissues are shown in **Table 4**, when the hens were fed with the MEL-contaminated diet for 34 consecutive days. MEL residues in hen tissues increased following the increase of MEL concentrations in feed. MEL concentrations were 21.6 ± 23.4, 9.3 ± 7.1, 6.9 ± 5.7, and 2.8 ± 1.9 μg/g in kidney, muscle, liver, and duodenum, respectively, when the hens were administered MEL at the level of 140.9 mg/kg of bw per day. These results distinctly show the distribution of MEL in hen tissues and that the highest MEL residue was in the kidney, followed by liver, muscle, ovary, uterus, and duodenum. Mast et al. have reported that the maximum amount of MEL residue was in the bladder, followed by the ureter, liver, and kidney when rats were administrated with a single oral dose of MEL (*3*). There was no significant difference of MEL concentrations in liver, duodenum, and kidney between all treatment groups ($p > 0.05$), which may result from the fact that MEL can be quickly excreted from the body. There were also not significant differences of MEL concentrations in plasma and all tissues between groups II, III, and IV ($p > 0.05$). However, MEL concentrations in plasma, uterus, muscle, and ovary from group VI were significantly higher than those from groups II, III, and IV.

Furthermore, the relationship between the MEL concentration in plasma and the MEL residue in kidney, muscle, liver, and masticatory stomach tissues of hens was best described by a series of quadratic functions. The coefficients of determination (R^2) of these quadratic functions were all greater than 0.97, which could be used to predict the residue in above tissues through determination of the MEL concentration in the plasma of the hens.

Elimination of MEL from Eggs. The MEL residue rapidly decreased in eggs when hens were withdrawn from the MEL-contaminated diets (**Table 5**). In our studies, the MEL concentration decreased to 30% on the second day and could not be detected by the HPLC−UV-based method on the seventh day following the MEL withdrawal. Only one egg from group IV contained detectable MEL at 0.4 μg/g on the fifth day, and 0.8 μg/g MEL was found in one egg from group VI, which were lower than the maximum residue limit of 2.5 μg/g, as announced by the Ministry of Health of the People's Republic of China in October 2008.

Elimination of MEL in Plasma and Tissues. There was no MEL residue in each tissue sample from hens of groups II, III, and IV on the 10th day following MEL withdrawal (**Table 6**). MEL residues were detected at 0.06 and 0.1 μg/g in liver and masticatory stomach tissues, respectively, from group V. Furthermore, there were 0.4, 0.5, 0.5, 0.6, and 0.1 μg/g MEL in the liver, masticatory stomach, ovary, intestine, and uterus, respectively, from group VI. However, MEL residues were completely eliminated for all treatment groups at 20 days after the MEL withdrawal. It was reported that MEL residues in kidney were higher and required more elimination time in the livers of pigs that were administered a MEL-contaminated diet (*31*). However, in this experiment, the MEL residues in kidney samples have greater

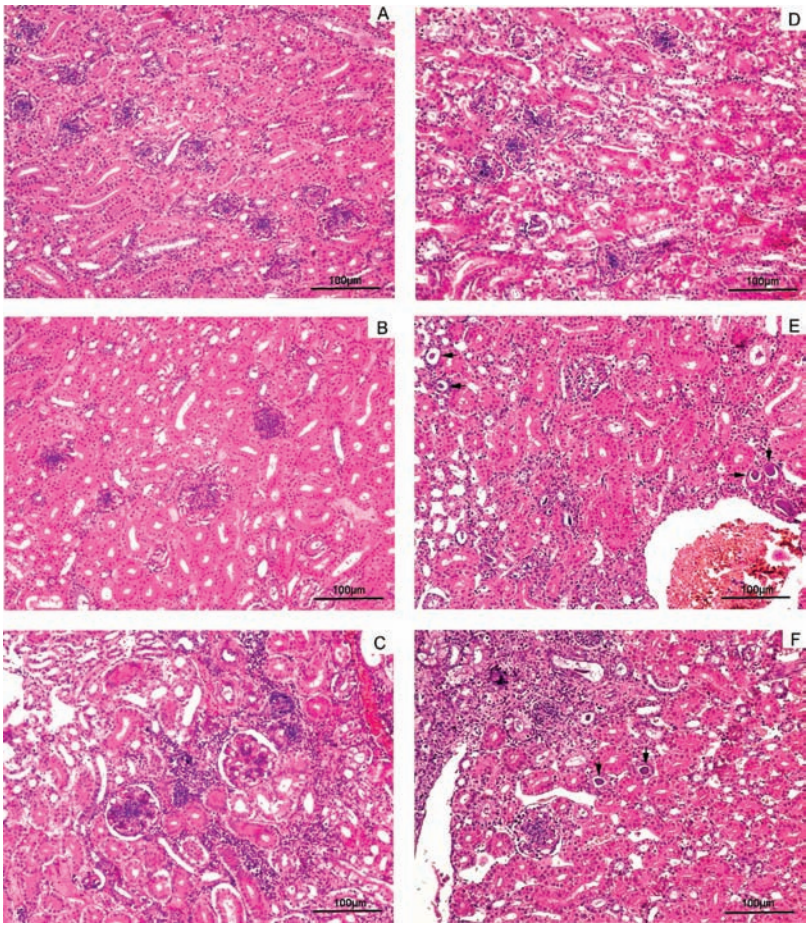

Figure 2. Photomicrographs of histologic preparations of sections of kidneys from hens that received MEL (8.6−140.9 mg/kg of bw) for 34 days and that were obtained 1 day after administration of MEL ceased. (A) Portion of kidney from the control group. (B) Portion of kidney from hens that received MEL at 8.6 mg/kg of bw for 34 days. Moderately pigmented tubules with empty lumens are evident, and this appearance was similar to that of kidneys from the control group. (C) Portion of kidney from hens that received MEL at 17.4 mg/kg of bw for 34 days. Some renal glomeruli in cortical area showed obvious swelling, and the small vessels in the interstitial tissue of the renal tubule exhibited vasodilatation. (D) Portion of kidney from hens that received MEL at 33.6 mg/kg of bw for 34 days. Dilated renal tubules and small blood vessel expansion are similar to that from kidneys of hens that received MEL at 17.4 mg/kg of bw. (E) Portion of kidney from hens that received MEL at 62.6 mg/kg of bw for 34 days. Crystals were dyed with royal purple in some renal tubule lumens. (F) Portion of kidney from hens that received MEL at 140.9 mg/kg of bw for 34 days. Crystals that was dyed with royal purple were also found. Bar =100 μm.

Table 3. Deposition of MEL in Eggs from Hens Exposed to Different Doses of MEL in the Basal Diet[a]

	concentration of MEL (μg/g)						
time (day)	group I (control)	group II (8.6 mg/kg of bw)	group III (17.4 mg/kg of bw)	group IV (33.6 mg/kg of bw)	group V (62.6 mg/kg of bw)	group VI (140.9 mg/kg of bw)	*p* value
1	NA[b]	NA	NA	NA	NA	NA	
2	ND[c]	1.1 ± 0.2 aA	2.1 ± 0.2 aA	5.6 ± 0.8 b	9.5 ± 1.4 c	21.5 ± 3.8 d	<0.001
3	ND	1.3 ± 0.2 aA	2.7 ± 0.4 aB	6.7 ± 0.4 b	10.5 ± 1.9 c	24.5 ± 4.4 d	<0.001
4	ND	1.5 ± 0.2 aA	2.9 ± 0.5 aB	5.5 ± 1.4 b	11.7 ± 2.3 c	26.5 ± 4.4 d	<0.001
5	ND	1.3 ± 0.2 aB	3.0 ± 0.5 aB	5.5 ± 0.9 b	11.1 ± 2.2 c	24.9 ± 4.2 d	<0.001
6	ND	1.3 ± 0.2 aB	2.8 ± 0.3 aB	5.6 ± 1.2 b	10.1 ± 2.7 c	23.9 ± 4.5 d	<0.001
7	ND	1.3 ± 0.1 aB	2.7 ± 0.4 aB	5.6 ± 1.2 b	11.0 ± 2.7 c	23.6 ± 4.5 d	<0.001
10	ND	1.5 ± 0.1 aB	3.0 ± 0.1 aB	5.9 ± 1.2 b	10.7 ± 2.1 c	28.7 ± 5.4 d	<0.001
14	ND	1.3 ± 0.2 aB	2.6 ± 0.3 aB	5.9 ± 1.1 b	8.4 ± 2.2 c	19.6 ± 2.2 d	<0.001
21	ND	1.6 ± 0.2 aB	2.8 ± 0.6 aB	6.5 ± 0.9 b	10.9 ± 1.5 c	24.1 ± 4.8 d	<0.001
28	ND	1.6 ± 0.3 aB	2.5 ± 0.5 aB	5.8 ± 0.8 b	11.6 ± 1.5 c	23.0 ± 4.6 d	<0.001
34	ND	1.4 ± 0.1 aB	2.8 ± 0.5 aB	5.81 ± 2.0 b	10.0 ± 2.0 c	23.1 ± 6.1 d	<0.001
p value		<0.001	0.03	0.648	0.458	0.172	

[a] The data in the table are mean ± standard deviation (SD) (n = 6). Different lowercase letters (a, b, c, and d) in the same row mean significant difference between the treatments ($p < 0.05$), and different capital letters (A and B) in the same column mean significant difference between treatments ($p < 0.05$). [b] NA = not applicable. [c] ND = not detectable.

elimination times than MEL residues in the liver, which may be due to the fact that hens are not mammals.

Although many countries have introduced limits for MEL concentrations in infant formula and other foods, little farm

Table 4. MEL Residues in Plasma and Tissues of Hens Exposed to MEL-Contaminated Diets for 34 Consecutive Days[a]

	concentration of MEL						
location	group I (control)	group II (8.6 mg/kg of bw)	group III (17.4 mg/kg of bw)	group IV (33.6 mg/kg of bw)	group V (62.6 mg/kg of bw)	group VI (140.9 mg/kg of bw)	*p* value
plasma	ND[b]	0.8 ± 0.2 a	0.9 ± 0.1 a	2.7 ± 1.9 ab	4.5 ± 2.0 bc	7.6 ± 3.7 c	0.004
liver	ND	0.5 ± 0.1	0.5 ± 0.1	1.5 ± 1.4	2.8 ± 1.5	6.9 ± 5.7	0.057
duodenum	ND	0.3 ± 0.1	0.3 ± 0.1	1.2 ± 1.1	0.7 ± 0.6	2.8 ± 1.9	0.198
masticatory stomach	ND	0.4 ± 0.1 a	0.6 ± 0.1 a	1.5 ± 1.6 a	3.5 ± 1.0 ab	7.3 ± 5.4 b	0.017
uterus	ND	0.5 ± 0.1 a	0.7 ± 0.1 a	1.2 ± 1.2 a	3.3 ± 1.0 a	6.9 ± 3.9 b	0.003
muscle	ND	0.4 ± 0.1 a	0.8 ± 0.3 a	1.6 ± 1.2 a	3.7 ± 1.7 a	9.3 ± 7.1 b	0.022
ovary	ND	0.5 ± 0.2 a	0.8 ± 0.1 a	1.7 ± 1.1 a	3.2 ± 0.9 a	9.1 ± 6.6 b	0.014
kidney	ND	1.3 ± 0.2	1.6 ± 0.8	2.7 ± 3.3	8.0 ± 5.0	21.7 ± 23.4	0.14

[a] The data in the table are mean ± SD ($n = 3$). The unit of MEL concentration in solid tissues is μg/g, and the unit of MEL concentration in plasma is μg/mL. Different lowercase letters (a, b, and c) in the same row mean significant difference between the treatments ($p < 0.05$), and same lowercase letter in the same row means no significant difference between treatments ($p < 0.05$). [b] ND = not detectable.

Table 5. Elimination of MEL in Eggs[a]

	concentration of MEL (μg/g)						
times (day)	group I (control)	group II (8.6 mg/kg of bw)	group III (17.4 mg/kg of bw)	group IV (33.6 mg/kg of bw)	group V (62.6 mg/kg of bw)	group VI (140.9 mg/kg of bw)	*p* value
1	ND[b]	1.1 ± 0.3	2.1 ± 0.1	4.7 ± 0.4	8.4 ± 0.6	15.7 ± 2.6	<0.001
2	ND	0.4 ± 0.1	1.0 ± 0.7	1.3 ± 0.1	2.9 ± 0.7	5.4 ± 1.4	<0.001
3	ND	0.2 ± 0.1	0.4 ± 0.1	0.8 ± 0.1	1.3 ± 0.3	2.4 ± 0.5	<0.001
4	ND	0.3 ± 0.1	0.3 ± 0.1	0.6 ± 0.2	1.1 ± 0.1	1.6 ± 0.3	
5	ND	ND	ND	0.4 ± 0.1[c]	ND	0.8 ± 0.2[c]	
7	ND	ND	ND	ND	ND	ND	

[a] The data in the table are mean ± SD ($n = 4$). [b] ND = not detectable. [c] Data are from one of four eggs.

Table 6. Elimination of MEL in Plasma and Tissues[a]

		concentration of MEL				
time (day)	locations	group II (8.6 mg/kg of bw)	group III (17.4 mg/kg of bw)	group IV (33.6 mg/kg of bw)	group V (62.6 mg/kg of bw)	group VI (140.9 mg/kg of bw)
10	plasma	ND[b]	ND	ND	ND	ND
	liver	ND	ND	ND	0.06 ± 0.01	0.4 ± 0.1
	kidney	ND	ND	ND	ND	ND
	muscle	ND	ND	ND	ND	ND
	masticatory stomach	ND	ND	ND	0.1 ± 0.05	0.5 ± 0.1
	uterus	ND	ND	ND	ND	0.1 ± 0.1
	ovary	ND	ND	ND	ND	0.5 ± 0.2
	duodenum	ND	ND	ND	ND	0.6 ± 0.2
20	plasma	ND	ND	ND	ND	ND
	liver	ND	ND	ND	ND	ND
	kidney	ND	ND	ND	ND	ND
	muscle	ND	ND	ND	ND	ND
	masticatory stomach	ND	ND	ND	ND	ND
	uterus	ND	ND	ND	ND	ND
	ovary	ND	ND	ND	ND	ND
	duodenum	ND	ND	ND	ND	ND

[a] The data in the table are mean ± SD ($n = 3$). The unit of MEL concentration in solid tissues is μg/g, and the unit of MEL concentration in plasma is μg/mL. [b] ND = not detectable.

animal feeding studies were available that would allow for a quantitative assessment of the carry-over of MEL into foods from animal feed (*32*). In this study, our experiments provided some information about MEL residues in eggs and hen tissues, as well as demonstrated the risks to human health posed by MEL.

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