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Effects of Melamine in Young Pekin Ducks

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Abstract: An experiment was conducted with male Pekin ducks to determine the toxicity of melamine (MEL) in young ducks fed dietary treatments from hatch to 21 days of age. Two hundred day-old male ducks were purchased from a commercial hatchery and assigned to one of ten treatment groups. Each treatment group consisted of five replicates with four ducks per replicate group. The diets contained 0.0, 0.25, 0.50, 0.75, 1.0, 1.25, 1.50, 1.75, 2.0 and 2.25% MEL. Compared with controls, ducks fed ≥ 1.0% MEL had reduced feed intake and body weight gain ($P<0.0001$). Compared to controls, relative kidney weights were higher ($P<0.0001$) in ducks fed diets containing ≥ 1.0% MEL. Compared with controls, ducks fed ≥ 0.25% MEL had increased ($P<0.0001$) kidney MEL concentrations, whereas ducks fed ≥ 0.75% MEL had increased ($P<0.0001$) muscle concentrations of MEL. MEL concentrations in the bile increased as dietary inclusion increased. Renal histopathology of all ducks with treatment related deaths were uniform with moderate to severe multifocal accumulation of eosinophilic to basophilic mineralized casts within renal tubules and collecting tubules. Histopathology results suggest that ≥ 1.5% MEL in the diet can cause severe renal pathology and mortality due to renal failure in ducks. The renal pathology observed in ducks was similar to that seen in other poultry species fed toxic concentrations of MEL.

Key words: Pekin duck, crystals, kidney, melamine toxicity

INTRODUCTION

Melamine ($C_3H_6N_6$; MEL) is a white, crystalline powder (OSHA, 2006) with a wide variety of industrial applications, including use in the manufacturing of plastics, adhesives, laminates, paints, flame retardants, textile finishes and fertilizers (Hilts and Pelletier, 2009). While MEL is not approved for use in animal feed (Lee *et al*., 2011) or human food (CDC, 2008) it is a known contaminant of both (Bhalla *et al*., 2009; Tyan *et al*., 2009). Contamination of feed or food can occur indirectly or accidentally by treatment of feed ingredients with products that contain MEL (WHO, 2008). Recently, the intentional adulteration of feeds and foods with MEL (Hilts and Pelletier, 2009) has received international attention. MEL is 66% nitrogen (Yang *et al*., 2009), therefore, protein analysis using the Kjeldahl method, will result in an invalid or an over estimation of the actual protein content of a matrix that contains MEL (Yang *et al*., 2009). For this reason, MEL was intentionally added to feed ingredients and foods to increase their monetary value (Cianciolo *et al*., 2008).

In 2007, deaths related to renal failure were documented in dogs and cats across North America and South Africa (Hilts and Pelletier, 2009). It was later determined that wheat gluten from China, used to manufacture the pet food, was intentionally contaminated with MEL and cyanuric acid (CA; Hilts and Pelletier, 2009). After examining six dogs and ten cats that had consumed contaminated pet food, Brown *et al*. (2007) noted that most had increased Blood Urea Nitrogen (BUN) and creatinine levels, with polarizable crystals present in the distal tubules and collecting ducts of the kidney.

Following the reports of contamination of pet food in 2007 it was determined that contaminated waste material from pet food manufacturing was incorporated into swine, poultry (Buur *et al*., 2008; USDA, 2007) and aquaculture feed (Karbiwnyk *et al*., 2009). Several studies were then conducted to determine the effects of MEL and CA, alone or in combination, on animal health and possible residue levels in meat destined for human consumption (Brand, 2011; Reimschuessel *et al*., 2008; Shen *et al*., 2010; Stine *et al*., 2011).

Brand (2011) conducted several studies on the effects of MEL and CA in young broilers and poults and reported that MEL significantly decreased the performance of broiler and poults, when included in the diet at ≥ 1.5%. Brand (2011) also found that the addition of CA to a diet contaminated with MEL reduced the toxic effects of MEL in poults but not in broilers.

In an experiment by Yan *et al*. (2009), ducks were fed graded levels of MEL ranging from zero to 1,000 mg/kg diet for 42 days. No MEL was detected in the breast, liver, or kidney of ducks consuming less than 50 mg/kg MEL (Yan *et al*., 2009). However, dietary levels of MEL

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between 100 and 1,000 mg/kg diet resulted in tissue residue levels that increased linearly as dietary levels increased, with ducks receiving 500 and 1,000 mg/kg diet having the highest concentrations of MEL in the kidneys (Yan *et al*., 2009).

Gao *et al*. (2010) fed laying ducks between zero and 100 mg/kg MEL and observed histological lesions in the kidneys of ducks fed ≥ 25 mg/kg MEL. Ducks fed 50 and 100 mg/kg MEL also had increased BUN levels. However, there was no effect on average egg weight, egg production, feed intake, or feed conversion when ≤ 100 mg/kg MEL was fed to the ducks.

The main objective of the current study was to determine the effects of MEL at concentrations ranging from 0 to 2.25%, in the diet of young Pekin ducks from hatch to 21 days. Two additional objectives were to determine if MEL accumulates in the tissues of Pekin ducks, and if hepatic clearance via bile is a possible route of MEL excretion.

MATERIALS AND METHODS

The animal care and use protocol was reviewed and approved by the University of Missouri-Columbia Animal Care and Use Committee (ACUC).

Diet preparation: A basal diet (Table 1) was formulated to meet or exceed all requirements of young Pekin ducks as suggested by the National Research Council (NRC, 1994). Ten dietary treatments were prepared by adding MEL purchased from Fisher Scientific to the basal diet. MEL was substituted for sand to obtain the desired dietary MEL concentrations of 0.0, 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, and 2.25%.

Table 1: Ingredient and nutrient composition of basal diet

Ingredient	Composition (%)
Corn	59.988
Soybean meal	34.774
Dicalcium phosphate	1.458
Limestone	0.507
Corn oil	0.451
Salt	0.333
Trace mineral[1]	0.100
Vitamin mix[2]	0.075
DL-methionine	0.060
Copper sulfate	0.004
Sand	2.250
Total	100.000
Nutrient composition (calculated)	
Crude protein (%)	22.00
Metabolizable Energy (kcal/Kg)	2, 900
Lysine (%)	1.19
Methionine (%)	0.40
Methionine+cysteine (%)	0.76
Threonine (%)	0.82
Calcium (%)	0.65
Non phytate phosphorus (%)	0.40

[1]Trace mineral mix provided (mg/kg of diet): Manganese, 110 mg from $MnSO_4$; iron, 60 mg from $FeSO_4 \bullet 7H_2O$; zinc, 110 mg from $ZnSO_4$; iodine, 2 mg from ethylenediamine dihydroiodide.

[2]Vitamin mix provided (per kg of feed): Vitamin A (retinyl acetate), 8, 800 IU; cholecalciferol, 3, 855 ICU; vitamin E (DL-α-tocopheryl acetate), 14 IU; niacin, 55 mg; calcium pantothenate, 17 mg; riboflavin, 6.6 mg; pyridoxine, 2.2 mg; menadione sodium bisulfate, 1.7 mg; folic acid, 1.4 mg; thiamin mononitrate, 1.1 mg; biotin, 0.2 mg; cyanocobalamin, 11 µg.

Birds, management and response variables: Two hundred day-old Pekin ducks were purchased from a commercial hatchery, weighed, leg banded and assigned to pens in stainless steel batteries. A completely randomized design was used, with five replicate pens of four ducks each assigned to each of the ten dietary treatments. Ducks were housed in an environmentally controlled room and placed on a 24-h constant light schedule. Feed and water were supplied for *ad libitum* consumption for 21 d. Ducks were observed daily and mortality was recorded as it occurred. All ducks that died before day 21 were weighed and sent to the avian pathology lab at the University of Missouri (Columbia, MO) for necropsy.

On day 21, ducks and feed were weighed and average body weight gain, average feed intake and feed conversion were calculated. All ducks were anesthetized with carbon dioxide and blood samples were collected via cardiac puncture from three birds per pen. Blood samples were centrifuged (Sorvall, RC 3 B plus) at 1,400 x g for 30 minutes at 7°C and serum was separated and frozen until analysis. Serum samples were analyzed for glucose (GLU), albumin (ALB), total protein (TP), globulin (GLOB), Aspartate Transaminase (AST), Gamma Glutamyltranserase (GGT) and uric acid (UA) at the University of Missouri Veterinary Clinical Laboratory (Columbia, MO). Following blood collection, ducks were euthanized by cervical dislocation. The liver and kidneys were removed and weighed from three ducks per pen. Relative liver and kidney weights were calculated by dividing organ weight by body weight. Sections of kidney from eight ducks per treatment were fixed in 10% neutral buffered formalin for histopathological evaluation. Samples of kidney, breast muscle and bile were collected from all treatments and frozen for later analysis of MEL concentrations.

Melamine analysis: Melamine extraction from tissue and bile samples was based on the method used by Brand *et al*. (2012) and involved High-performance Liquid Chromatography (HPLC) with UV detection. For kidney and muscle, 10 mL of water:Acetonitrile (1:2) was added to 1 g of tissue and the tissues homogenized (Bio Homogenizer, model M133/1281-0, Biospec Products Inc. Bartlesville OK 74005) for 30 sec in a 50 mL conical centrifuge tube. The homogenized sample was then centrifuged for 5 min at 1,000 rpm (Dynac II centrifuge) and the supernatant transferred to microcentrifuge tubes and further centrifuged for 5 min at 10,000 rpm (Spectrafuge 16M). The supernatant was extracted and passed through a MycoSep® 224 AflaZon column (Romer Labs, 2011). Finally, 500 µL of the filtered

supernatant was diluted (1:1) with buffer solution (BUFF; 1.924 g citric acid and 2.34 g of octanesulfonate dissolved in 1 L of distilled water, pH adjusted to 3 using NaOH) before HPLC analysis was performed.

For bile, extraction involved adding 200 µL of bile to 1,800 µL of water:acetonitrile (1:2), vortexing and transferring the samples to microcentrifuge tubes and centrifuging for 5 min at 10,000 rpm (Spectrafuge 16M). The supernatant was collected and passed through a MycoSep® 224 AflaZon column (Romer Labs, 2011). Finally, 500 µL of the eluant was diluted (1:1) with BUFF before high-performance liquid chromatography (HPLC) analysis was performed.

A Hitachi Model L-7100 pump with a Model L-7485 UV detector, Hitachi Model L-7200 autosampler with Hitachi D-7000 data acquisition interface and ConcertChrom software on a microcomputer were used for HPLC analysis. A HyperClone (Phenomonex) C_{18} column (100 x 4.60 mn; 3 µm) was used with a retention time of 6 min and flow rate of 1 mL/min. UV detection was at 240 nm. The mobile phase consisted of BUFF:acetonitrile(ACN; 87:13).

A primary standard of 2,000 ppm MEL solution was diluted with BUFF:ACN (1:1) to prepare standards of 1, 5, 10 and 20 ppm MEL. MEL standards were ran before and after each set of samples and used to calculate a standard curve. The area under the peak was calculated, plotted on the standard curve, and used to calculate individual MEL concentrations in samples.

Statistical analysis: Data were analyzed using the general linear model procedures of Statistical Analysis Software (SAS) (SAS, 2006). Pen was considered the experimental unit. Variables that showed significant differences in the ANOVA were compared using Fisher's protected least significant difference procedure (SAS, 2006). Regression analysis was performed on all data to determine linear ($y_i = a+bx_i+E_i$) or quadratic ($y_i = a+bx_i+cx^2_i+E_i$) responses. Statistical significance was accepted at a P-value of $\leq$ 0.05. An arcsine transformation was applied to percent mortality data and a log10 transformation applied to kidney MEL residue data before statistical analysis was performed.

RESULTS

Performance and mortality: The effects of MEL on body weight gain, feed intake, feed conversion, and mortality are summarized in Table 2. Feed intake and body weight gain decreased linearly (P<0.0001) with increasing dietary concentrations of MEL, whereas feed conversion increased quadratically (P<0.003) with increasing dietary MEL concentrations. Compared to controls, birds fed $\geq$ 1.00% MEL had decreased (P<0.0001) feed intake and body weight gain, whereas birds fed $\geq$ 1.50% MEL had poorer (P<0.0001) feed conversion than controls. Percent mortality also increased in a quadratic (P<0.01)fashion with increasing dietary MEL concentrations. Compared to controls, mortality was higher (P<0.001) in ducks that consumed $\geq$ 2.00% MEL.

Organ weights: Table 2 summarizes the effects of MEL on relative organ weights. Relative liver weights of ducks responded in a quadratic (P<0.005) fashion with increasing dietary MEL concentrations. Relative kidney

Table 2: Effects of melamine (MEL) on performance and organ weights of Pekin ducks

Treatments[1]		Response variables[2]					
Melamine (%)		BWG (g)	FI (g)	F:G (g:g)	Mortality[3] (%)	Liver[4] (%)	Kidney[4] (%)
Basal diet+0.00% MEL		832^{a}	1, 658^{a}	2.01^{d}	5^{bc}	3.89	1.18^{d}
Basal diet+0.25% MEL		787^{a}	1, 647^{a}	2.09^{d}	0^{c}	3.84	1.20^{d}
Basal diet+0.50% MEL		774^{a}	1, 590^{a}	2.05^{d}	0^{c}	4.04	1.32^{cd}
Basal diet 0.75% MEL		775^{a}	1, 557^{a}	2.04^{d}	5^{bc}	4.08	1.26^{cd}
Basal diet+1.00% MEL		615^{b}	1, 324^{b}	2.17^{d}	0^{c}	4.23	1.95^{bc}
Basal diet+1.25% MEL		615^{b}	1, 361^{b}	2.22^{d}	0^{c}	4.12	1.94^{c}
Basal diet+1.50% MEL		458^{c}	1, 128^{c}	2.65^{c}	15^{ab}	4.00	2.64^{ab}
Basal diet+1.75% MEL		439^{cd}	1, 146^{c}	2.73^{bc}	5^{bc}	3.97	2.84^{a}
Basal diet+2.00% MEL		358^{d}	1, 028^{c}	3.12^{a}	20^{a}	3.67	3.30^{a}
Basal diet+2.25% MEL		392^{cd}	1, 052^{c}	3.02^{ab}	20^{a}	3.71	3.33^{a}
ANOVA:[5]	S.E.M.	32	59	0.11	0.08	0.15	0.24
	P-Value	<0.0001	<0.0001	<0.0001	0.0008	0.2104	<0.0001
Regression:[6]	L: P-Value	<0.0001	<0.0001	<0.0001	<0.0001	0.1879	<0.0001
	Q: P-Value	0.6289	0.9144	0.0023	0.0119	0.0034	0.0952

[1]Treatments were the addition of melamine, in percent indicated, to a basal diet. Diets were fed for 21 consecutive days.

[2]Data are means of five replicate pens, with four ducks per pen for BWG, AFI, F:G and Mortality and three ducks per pen for relative liver and kidney weights.

[3]Means are percent of mortality that occurred out of 20 birds. Statistical analysis was performed on transformed data (arcsine).

[4]Relative organ weights, expressed as a percentage of body weight.

[5]One way analysis of variance values.

[6]Regression: Linear (L) or quadratic (Q) regression.

[a-d]Means within a column with no common superscript are different (P<0.05).

BWG = average body weight gain; FI = average feed intake; F:G = feed to gain.

Table 3: Effects of melamine (MEL) on serum chemistries of Pekin ducks

Treatment[1]		Response variables[2]						
Melamine (%)		GLU (mg/dL)	ALB (g/dL)	TP (g/dL)	GLOB (g/dL)	AST (U/L)	GGT (U/L)	UA (mg/dL)
Basal diet+0.00% MEL		224	1.32^{c}	3.12^{c}	1.82^{c}	53.4	2.68^{de}	7.34^{d}
Basal diet+ 0.25% MEL		226	1.32^{c}	3.10^{c}	1.78^{c}	36.9	2.32^{e}	8.62^{d}
Basal diet+0.50% MEL		184	1.34^{bc}	3.26^{c}	1.90^{c}	38.5	2.48^{e}	7.78^{d}
Basal diet+0.75% MEL		199	1.34^{bc}	3.30^{c}	1.98^{c}	28.0	2.54^{e}	11.56^{cd}
Basal diet+1.00% MEL		263	1.62^{a}	3.96^{ab}	2.40^{ab}	29.5	3.86^{bcd}	25.10^{ab}
Basal diet+1.25% MEL		239	1.50^{ab}	3.80^{b}	2.30^{b}	73.4	3.00^{cde}	21.44^{bc}
0Basal diet+1.50% MEL		252	1.50^{ab}	3.98^{ab}	2.46^{ab}	99.7	4.02^{bc}	31.62^{ab}
Basal diet+1.75% MEL		248	1.54^{a}	4.10^{ab}	2.54^{a}	57.3	3.86^{bcd}	35.48^{a}
Basal diet+2.00% MEL		255	1.58^{a}	4.10^{ab}	2.52^{a}	239.8	4.74^{ab}	33.84^{a}
Basal diet+2.25% MEL		270	1.60^{a}	4.18^{a}	2.58^{a}	188.6	5.44^{a}	34.74^{a}
ANOVA:[3]	S.E.M	25	0.06	0.12	0.08	52.2	0.45	4.18
	P-Value	0.2825	0.0002	<0.0001	<0.0001	0.0733	<0.0001	<0.0001
Regression:[4]	L: P-Value	0.0193	<0.0001	<0.0001	<0.0001	0.0021	<0.0001	<0.0001
	Q: P-Value	0.6393	0.4101	0.1555	0.1016	0.0609	0.0710	0.6567

[1]Treatments were the addition of melamine, in percent indicated, to a basal diet. Diets were fed for 21 consecutive days.
[2]Data are means of five replicate pens with three ducks per pen.
[3]One way analysis of variance values.
[4]Regression: Linear (L) or quadratic (Q) regression.
[a-e]Means within a column with no common superscript are different (P<0.05).
GLU = glucose; ALB = albumin; TP = total protein; GLOB = globulin; AST = asparate transaminase; GGT = gamma glutamyltranserase; UA = uric acid.

Table 4: Melamine (MEL) concentrations in kidney, muscle and bile of Pekin ducks

Treatment[1]		Response variables		
Melamine (%)		Kidney[2] (mg/kg)	Breast Muscle[3] (mg/kg)	Bile[4] (mg/kg)
Basal diet+0.00% MEL		ND^{e}	ND^{f}	ND
Basal diet+0.25% MEL		29^{d}	14^{f}	25
Basal diet+0.50% MEL		42^{c}	13^{f}	91
Basal diet+0.75% MEL		161^{b}	74^{e}	203
Basal diet+1.00% MEL		335^{a}	127^{d}	475
Basal diet+1.25% MEL		284^{a}	135^{cd}	427
Basal diet+1.50% MEL		385^{a}	212^{b}	613
Basal diet+1.75% MEL		388^{a}	184^{bc}	575
Basal diet+2.00% MEL		340^{a}	173^{bcd}	640
Basal diet+2.25% MEL		295^{a}	262^{a}	560
ANOVA[5]:	S.E.M	0.05	17	-
	P-value	<0.0001	<0.0001	-
Regression[6]:	L: P-value	<0.0001	<0.0001	-
	Q: P-value	<0.0001	0.4728	-

[1]Treatments were the addition of melamine, in percent indicated, to a basal diet. Diets were fed for 21 consecutive days.
[2]Data are means of four replicate pens, with one duck per pen. Statistical analysis for kidney was performed on transformed data (log10).
[3]Data are means of four replicate pens, with one duck per pen.
[4]Bile from all replicates was pooled and data are means of duplicate HPLC analysis per treatment. Statistical analysis not performed due to lack of replicates.
[5]One way analysis of variance values.
[6]Regression: Linear (L) or quadratic (Q) regression.
[a-f]Means within a column with no common superscript are different (P<0.05). ND: None detected.

weights increased linearly (P<0.0001) with increasing dietary concentrations of MEL, with ducks fed = to 1.00% MEL having heavier (P<0.0001) relative kidney weights than control ducks.

Serum chemistry: The effects of MEL on the serum chemistry of young Pekin ducks are summarized in Table 3. Serum GLU (P = 0.02), ALB (P<0.0001), TP (P<0.0001), GLOB (P<0.0001), AST (P<0.003), GGT (P<0.0001) and UA (P<0.0001) all increased linearly with increasing dietary concentrations of MEL. Compared to controls, serum ALB, TP, GLOB and UA were all higher (P<0.001) in ducks fed $\geq$ 1.00% MEL, whereas serum GGT (P<0.0001) was higher in ducks fed 1.5% MEL and in ducks fed $\geq$ 2.0% MEL when compared to controls.

Tissue residues: Table 4 shows residue levels of MEL in the kidney, breast muscle and bile as determined by

HPLC. MEL concentrations in breast muscle increased linearly ($P<0.0001$) with increasing dietary concentrations of MEL, whereas MEL concentrations in kidney increased quadratically ($P<0.0001$). Ducks fed $\geq$ 0.25% MEL had MEL concentrations in the kidneys that were higher ($P<0.0001$) than levels found in controls, whereas ducks fed $\geq$ 0.75% MEL had MEL concentrations in breast muscle that were higher ($P<0.0001$) than in controls. However, at each MEL inclusion level, MEL concentrations were lower in the breast muscle than in the kidney.

Due to logistical problems during termination, bile from ducks in each treatment was pooled. Therefore, statistical analysis could not be performed on the bile data. Means presented in Table 4 are averages of duplicate HPLC analysis performed on bile samples taken from each treatment. Melamine concentrations in the bile increased as dietary MEL concentrations increased, with a low of 25 mg/kg in ducks fed 0.25% MEL and a high of 640 mg/kg in ducks fed 2.00% MEL.

Gross pathology-mortality: Table 2 showed the percent mortality that occurred in each treatment group. In total, 14 ducks died over the 21 day experimental period. Two mortalities, one in the control and one in the 0.75% MEL group, were the results of trauma and hemorrhaging and were unrelated to dietary treatments. The three mortalities in the 1.50% MEL treatment group had pale and enlarged kidneys with little to no feed in the upper gastrointestinal tract. One of these three ducks also had crystals in its bile. The one mortality that occurred in the 1.75% MEL treatment group presented with pale and enlarged kidneys. The 2.00 and 2.25% MEL treatment groups had four mortalities each. All but one duck from the 2.00 and 2.25% MEL groups had pale or pale and enlarged kidneys, with little to no feed in the upper gastrointestinal tract. At time of death, white crystals were present in the bile of three of the mortalities from the 2.00 and 2.25% MEL groups. Figure 1 shows crystals present in the bile of a duck fed a diet with MEL (photograph 'B') and photograph 'A' shows the clear bile from a duck fed the control diet.

Gross pathology-termination: By the end of the 21 day study there was a clear size difference among ducks from the control group and ducks fed diets containing high levels of MEL. Gross examination of ducks that received diets with high levels of MEL ($\geq$ 1.00% MEL) revealed enlarged and pale kidneys.

Histopathology-early mortality: Histopathologic examination of the kidneys from early mortalities occurring in the $\geq$ 1.50% MEL groups revealed moderate to severe autolysis in all sections with numerous basophilic mineralized casts noted within the renal

Fig. 1: Effect of melamine (MEL) on the bile of young Pekin ducks fed treatments from hatch to 21 days of age. A) Bile from a duck fed the control diet. B) Bile from a duck fed MEL. Cloudy appearance of bile in photo 'B' is due to presence of white crystal precipitate

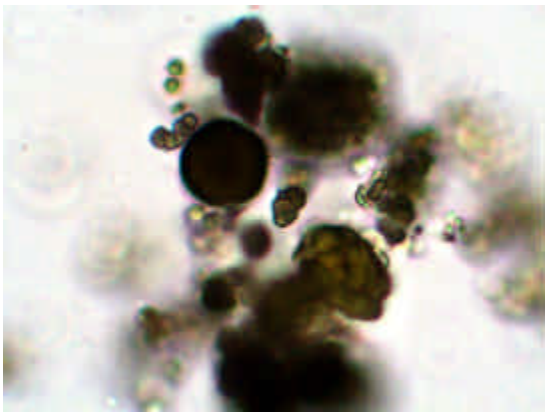

Fig. 2: Microscopic examination of bile from ducks fed graded levels of melamine (MEL). Crystals observed at 400 x magnification in the bile of duck fed 2.25% MEL for 21 days

tubules and collecting ducts. The renal lesions noted in these mortalities were compatible with MEL toxicity seen in other avian species.

Histopathology-termination: Histopathology of kidney sections from ducks fed 0.00, 0.25 and 0.50% MEL were unremarkable. Two kidneys from the 0.75% MEL treatment group had mild dilation of the embryonal

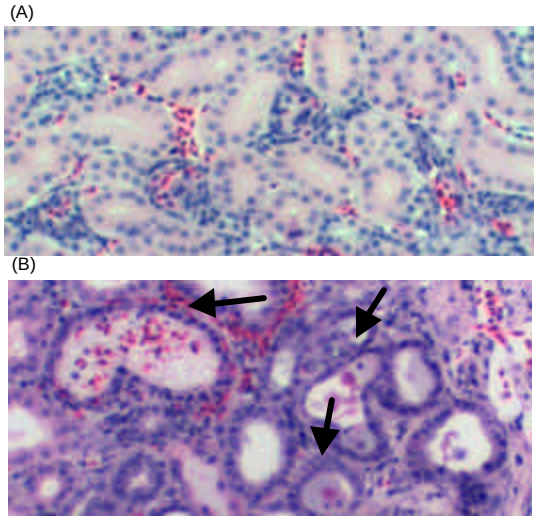

Fig. 3: Kidney section from a control and duck fed 2.25% melamine (MEL), viewed at 100 x magnification. Photo 'A' shows the appearance of a normal kidney section while photo 'B' shows the effects of 2.25% MEL on the kidney of a young Pekin duck from hatch to 21 days of age. Arrows point to dilated tubules with casts present

nephrons with eosinophilic to basophilic casts present. Several kidney sections from each treatment fed ≥ 1.00% MEL contained eosinophilic to basophilic casts, with some casts containing spherical crystals. The incidence of crystals increased as percent MEL in the diet increased. Crystals were also present in the interstitial spaces with mild multifocal heterophil infiltration present in kidney sections of ducks fed ≥ 1.00% MEL (Fig. 3). The previously described pathology is compatible with mild MEL toxicity. In addition to the previously described findings, several kidneys from ducks fed ≥ 2.00% MEL had moderate to severe dilation of embryonal nephrons, collecting tubules and urinary space of the glomeruli. These finding suggest mild to severe MEL toxicity in treatments fed ≥ 2.00% MEL.

Microscopic examination of bile samples: Microscopic examination of pooled bile specimens collected during termination revealed a moderate number of one to ten µm spherical brown crystals with numerous aggregated crystals in treatments fed ≥ 1.00% MEL. Bile from ducks fed 2.25% MEL had crystals that measured one to 20 µm with identical appearance as crystals in the bile of ducks fed ≥ 1.00% MEL. The crystals observed in the bile of treatments fed ≥ 1.00% MEL exhibited birefringence when viewed under polarized light. Figure 2 shows crystals present in the bile of ducks fed 2.25% MEL for 21 days.

DISCUSSION

The concentration of MEL (≥ 2.00%) that caused significant mortality in ducks was intermediate between the concentrations reported to cause mortality in poults (≥ 1.5%) and broilers (≥ 2.5%) by Brand (2011). However, it should be noted that 15% mortality was observed in ducks fed 1.50% MEL, and although this level of mortality was not significantly different from controls, examination of the kidneys of these ducks revealed lesions compatible with MEL toxicity.

The reduction in body weight gain and feed intake in ducks that consumed diets containing ≥ 1.00% MEL is consistent with previous reports by Brand (2011) who also observed reductions in feed intake and body weight gain of broilers and poults that consumed ≥ 1.00% MEL. The ability of ducks to convert feed to gain was reduced when ≥ 1.50% MEL was included in the diet. Brand (2011) did not observe an increase in feed to gain in broilers until dietary MEL levels were ≥ 2.5% and no changes in feed conversion were observed in poults fed up to 1.50% MEL. Gao *et al.* (2010) fed up to 100 mg/kg (0.01% of the diet) MEL to laying ducks for 42 days and did not observe any negative effects on body weight gain, feed intake, or feed conversion. Data from the current study shows that ≤ 0.75% MEL in the diet did not reduce performance of young Pekin ducks. Relative liver weights of ducks increased at dietary MEL concentrations ranging from 0.50 to 1.75% then decreased at MEL concentrations ≥ 2.00% resulting in a quadratic response. Brand (2011) report heavier relative liver weights in young broilers fed ≥ 2.25% MEL and a linear increase in relative liver weight was also observed in poults fed up to 1.5% MEL (Brand, 2011).

Relative kidney weight increased linearly as dietary MEL concentrations increased, and ducks fed ≥ 1.00% MEL had heavier kidneys than the control ducks. Increased relative kidney weight were also observed in broilers fed ≥ 1.50% MEL and poults fed ≥ 1.00% MEL (Brand, 2011). Gao *et al.* (2010) fed 100 mg/kg MEL (0.01% of the diet) to laying Jinding ducks and also observed heavier relative kidney weights after 21 days on test. The increase in relative kidney weight in laying ducks, at a much lower concentration of MEL than that observed in young Pekin ducks, suggests variability in how different types and ages of ducks are affected by MEL. However, it appears that young Pekin ducks are affected in a similar manner to young broilers and poults, with ≥ 1.00% MEL required to cause a significant increase in relative kidney size.

All serum metabolites measured (GLU, ALB, TP, GLOB, AST, GGT, and UA) were found to be increased above levels found in control birds and/or were found to increase in a linear fashion as dietary MEL concentrations increased. Brand *et al.* (2012) observed

increased serum ALB, TP, GLOB, AST and GGT above levels of control birds, in broilers fed graded levels of MEL up to 3.00% of the diet. No changes in serum chemistry were observed in poults fed up to 1.50% MEL (Brand, 2011). However, it should be noted that high treatment related mortality occurred in poults fed 2.00, 2.50, and 3.00% MEL, and as a result serum samples were not available for analyses. Elevated UA can be used to diagnose renal failure, with increased levels occurring when more than 70% of kidney function is lost (Cornell, 2010). In the present study, ducks consuming 2.25% MEL had UA levels that were 4.7 times that of control ducks. Brand (2011) reported that UA levels in broilers increased in a linear fashion with increasing dietary MEL levels. However, no difference was reported between controls and broilers fed up to 3.0% MEL or poults fed up to 1.5% MEL for 21 days (Brand, 2011).

Melamine concentrations in the kidney increased in a quadratic fashion with ducks fed $\geq$ 0.25% MEL having higher concentrations of MEL in the kidney compared to that of control ducks. Compared to kidney MEL concentrations of ducks fed 2.25% MEL for 21 days in the current study (295 mg/kg), kidney MEL concentrations of broilers and turkeys fed 2.25% MEL for 21 days were 2.8 (846 mg/kg) and 2 (586 mg/kg) fold higher, respectively (Brand *et al*., 2012). This two to 2.8 fold difference among broilers, turkeys and ducks could be due to differences in how the three species metabolize MEL especially with respect to absorption and excretion. Lower absorption and or greater excretion of MEL would result in a lower accumulation of MEL in the tissues in particular the kidney which is the target organ for MEL.

Concentrations of MEL in muscle tissue were lower than MEL concentrations in the kidney. This difference in MEL concentrations between muscle and kidney is similar to a previous report by Lu *et al*. (2009), in which the kidneys of broilers fed 0.1% MEL for 42 days had a MEL concentration of 9.17 mg/kg compared to 3.73 mg/kg in the breast meat. Higher MEL concentrations in the kidney compared to breast muscle is supported by data from Dobson *et al*. (2008) and Puschner *et al*. (2007) who noted precipitation of MEL and CA complexes in the kidney. Precipitation of MEL and CA complexes in the kidney probably occur because of increased concentrations of the compounds as they move down the osmotic gradient (Dobson *et al*., 2008). Therefore, it is reasonable to assume that MEL concentrations in the kidney would be greater than that in the muscle, due to the compound becoming more concentrated by the function of the kidneys. Another possible explanation for the difference in MEL concentrations between the muscle and kidney could be the relative mass of the two tissues. The greater mass of muscle tissue could have contributed to a dilution effect resulting in a lower concentration.

In the current study, bile had a much higher concentrations of MEL than the kidney, suggesting that bile is a route of MEL excretion in Pekin ducks. Use of the bile as a route of excretion has been suggested to occur in broilers and poults by Brand (2011) . However, it remains to be determined if biliary excretion occurs only at high concentrations, such as those used in the current study, or also at lower dietary concentrations of MEL.

Gross and histopathology of early mortalities revealed that birds fed $\geq$ 1.50% MEL were off feed at time of death. All but one of these ducks had pale and enlarged kidneys. Enlarged and pale kidneys have also been observed in poults and broilers fed $\geq$ 1.00 and $\geq$ 2.00% MEL, respectively (Brand, 2011). The renal lesions noted in early mortality ducks were comparable to those seen in broilers and poults fed toxic concentrations of MEL (Brand, 2011).

Gross pathology of ducks that survived to termination revealed similar findings as early mortality ducks, with pale and enlarged kidneys. Histopathology revealed that MEL did not cause renal damage until dietary MEL inclusion was $\geq$ 0.75%. Dietary MEL levels between 0.75% to 1.75% caused renal damaged compatible with mild toxicity. Dietary MEL levels $\geq$ 2.00% caused renal damage compatible with mild to severe toxicity. The pathology documented in ducks fed $\geq$ 0.75% MEL is comparable to that reported in broilers and poults (Brand, 2011). Mature Jinding ducks fed 25 mg/kg MEL (0.0025% of the diet) for 21 days showed signs of tubular cell necrosis and lymphocytic infiltration of the kidneys during histological examination (Gao *et al*., 2010). Reasons for the greater sensitivity of mature Jinding ducks to MEL as compared to young Pekin ducks are unknown at this time and require further investigation.

It appears that concentrations of MEL in the bile need to be in excess of 203 mg/kg to favor crystal formation. This observation is based on the levels of MEL detected in the bile via HPLC analysis. The bile of ducks fed 0.75% MEL had a MEL concentration of 203 mg/kg with no crystals visible during microscopic examination, whereas ducks fed 1.00% MEL had bile MEL concentration of 475 mg/kg with a moderate number of crystals present in the bile.

Histopathologic evaluation revealed that 1.00% MEL was the lowest dietary treatment to induce crystal formation in the kidney. At this level, HPLC analysis determined a MEL concentration of 335 mg/kg in the kidney. The next lowest treatment, 0.75% MEL, had a kidney MEL concentration of 161 mg/kg and with no crystals detected. This suggests that MEL concentrations in the kidney need to exceed 161 mg/kg to induce crystal formation in the kidney.

Conclusion: There are documented changes in the gross renal appearance and findings of the histological

examination that are indicative of renal damage. These findings coupled with elevated levels of GLU, ALB, TP, GLOB, AST, GGT and UA indicate decreased renal function suggesting that renal failure induced by MEL damage is the most probable cause of the decreased performance and mortality noted during the current study. With significant decreases in body weight gain, feed intake and increases in relative kidney weights occurring in ducks fed $\geq$ 1.00% MEL, along with changes in serum ALB, TP, GLOB and UA, at the same level, it appears that dietary concentrations $\geq$ 0.75% can be tolerated, without significant negative effects, in young Pekin ducks. Results also indicate that at these dietary concentrations of MEL, the bile is used as an excretory route by ducks. Although the lower levels of MEL did not cause toxicity, it should be noted that the addition of MEL to diets is illegal and subject to criminal prosecution in the United States.

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