EFFECTS OF DIETARY MELAMINE AND CYANURIC ACID IN YOUNG PEKIN DUCKS AND WEANLING PIGS

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The undersigned, appointed by the Dean of the Graduate School, have examined the thesis entitled

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Presented by Benjamin Robert Landers

A candidate for the degree of Master of Science

And hereby certify that in their opinion it is worthy of acceptance.

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EFFECTS OF DIETARY MELAMINE AND CYANURIC ACID IN YOUNG PEKIN DUCKS AND WEANLING PIGS

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ABSTRACT

In 2007 the intentional contamination of feed ingredients with melamine (MEL) to artificially increase the calculated crude protein and thus monetary value of feed ingredients lead to the deaths of cats and dogs across North America. It was later documented that waste material from contaminated pet food was fed to swine and poultry across the United States. To determine the effects of feeding MEL and cyrnauric acid (CYA), a structural analog of melamine, three experiments were conducted to determine the individual and combined effects of MEL and CYA on young Pekin ducks and weanling pigs. In young Pekin ducks ≥ 1.00 % MEL caused a decrease in performance and changes in serum chemistry values indicating renal failure. Up to 1.50% CYA did not affect performance of young Pekin ducks and the addition of CYA to diets containing MEL alleviated the negative effects of MEL. Lesions such as pale and enlarged kidneys with crystals present in the lumina of the collecting ducts/tubules detected in ducks fed ≥ 1.00 % MEL were similar to lesions documented in broilers, poults, and cats. In young barrows, up to 1.25 % MEL did not cause changes in blood urea nitrogen or creatinine levels, renal pathology, or mortality during the treatment period. However, levels ≥ 1.00 % MEL did cause a reduction in body weight gain over the 21 day experimental period. However, the combination of 0.75% MEL + 0.75% CYA caused barrows to have

lower body weight gains than controls and higher blood urea nitrogen and creatinine levels than controls. From the MEL residue data collected it appears that bile is a route of elimination of MEL used by young Pekin ducks and weanling pigs (barrow). Samples of kidney, muscle, and bile from the ducks and pigs were analyzed for MEL residue levels via high-performance liquid chromatography (HPLC). Results of HPLC analyses revealed the bile to have the highest concentration of MEL followed by the kidney and finally the muscle, in both the duck and pig. The HPLC data also suggests that ingestion of a combination of MEL and CYA leads to precipitation of a MEL-CYA complex in the gastrointestinal tract that decreases the absorption of MEL into the body. This reduced absorption reduces MEL residue levels in the muscle, bile, and kidney of ducks and barrows.

CHAPTER 1

Introduction

In March of 2007, deaths related to renal failure were documented in dogs and cats across North American and South Africa (Hilts and Pelletier, 2009). It was later determined that wheat gluten imported from China and incorporated into the pet food was contaminated with melamine and related compounds (cyanuric acid, ammeline, and ammelide) (Byungghul et al., 2008). By April of 2007 some 100 different brands of dog and cat food had been voluntarily recalled across the United States (FDA, 2007). Approximately four months later the American Association of Veterinary Laboratory Diagnosticians reported that some 347 cases met the criteria for "pet food-induced nephrotoxicity" based on high concentrations of blood urea nitrogen and creatinine (Burns, 2007).

Following the contamination of pet food, it was determined that waste material containing melamine and/or cyanuric acid from pet food manufacturing was incorporated into swine, poultry (Buur et al., 2008; Karbiwnyk et al., 2009; USDA, 2007a) and aquaculture feeds (Karbiwnyk et al., 2009). After contamination of feeds with melamine and related compounds was confirmed, all animals that had consumed the adulterated feed were placed under quarantine (USDA, 2007a). However, after the testing of muscle tissue and urine from these animals, USDA announced that poultry and swine products

from the animals were “not adulterated” and were able to be offered for slaughter (USDA, 2007a).

Since the 2007 incidence of pet food contamination with melamine and related analogs, several case reports and experiments have been published (Baynes et al., 2008; Brand, 2011; Brown et al., 2007; Gonzalez et al., 2009; Reimschuessel et al., 2008; Stine et al., 2011). In 2008, there were documentations of children in China treated for renal complications after having consumed infant formula contaminated with melamine (Chan et al., 2008; WHO, 2008a). Brand (2011) did considerable work on the individual and combined effects of melamine and cyanuric acid in young broilers and poults. During this Brand (2011) found that the addition of cyanuric acid to a diet containing melamine caused less toxicity in poults than diet contaminated with only melamine. These results are in contrast to results in mammals (dogs, cats, and pigs), where the combination of the two compounds was found to be more toxic than either compound alone (Puschner et al., 2007; Reimschuessel et al., 2008; Stine et al., 2011).

The objectives of the current thesis were to document the individual and combined effects of melamine and cyanuric acid in young Pekin ducks and weanling pigs (barrows). Additionally, the studies presented in the current thesis were used to determine melamine concentrations in the kidney, muscle, and bile of young Pekin ducks and weanling pigs fed dietary treatments for 21 days. A final objective was to determine if mammals used bile as a route to eliminate melamine from their bodies; a process reported to occur in poultry by Brand (2011).

CHAPTER 2

Literature Review

Chemical Properties and Applications:

Melamine (2,4,6-triamino-1,3,5-triazine) is a white, crystalline powder (OSHA, 2006; SafetyData, 2008) that was first synthesized by Liebig in 1834 (Zhang et al., 2007). It is a small polar molecule (Baynes et al., 2008) with a molecular formula of $C_3H_6N_6$ (OSHA, 2006; SafetyData, 2008). Due to the presence of several basic amino groups it has a PK_b of 9.0 (Baynes et al., 2008) and melting point of 345º C (SafetyData, 2008). Figure 2.1. depicts the structure of melamine with its three ring N atoms and three amino groups which enables melamine to act as both a hydrogen-bond donor and acceptor (Zhang et al., 2007), thus enabling it to form extensive hydrogen bonds (Colombo et al., 1985).

Commercial production of melamine involves heating dicyandiamide or urea in the presence of ammonia (Tyan et al., 2009). Bizzari and Yokose (2008) reported that approximately 1.2 million tonnes of melamine was produced worldwide in 2007, with China being the largest producer and consumer. Applications of melamine include use in manufacturing of plastics, adhesives, laminates, paints, permanent-press fabrics, flame retardants, textile finishes, tarnish inhibitors, paper coating, and fertilizer mixtures (Hilts and Pelletier, 2009). Cyromazine is an example of a chemical insecticide that contains melamine and acts as an insect growth regulator (Chou et al., 2003). It is used for feed-

through fly control in caged layers (Chou et al., 2003) and is usually incorporated into laying hen diets at 0.50 parts per million (ppm) (Agrochemicals, 1993). Though it is a highly effective pesticide, it can potentially be degraded to melamine (Chou et al., 2003).

While melamine has wide spread use in manufacturing, fertilizers, and pesticides, it is not approved for use in animal feed (Lee et al., 2011) or human food (CDC, 2008); however, melamine is a known contaminant of both (Bhalla et al., 2009; Tyan et al., 2009). Contamination of food and feeds can occur through several different routes including: 1) trace levels of melamine found in the environment contaminating the food chain; 2) the accidental contamination of animal feed or human food that have been treated with products that contain melamine, such as fertilizer and pesticides; 3) the leaching of melamine monomers from plastic and tableware products; 4) or adulteration which is "the intentional addition of melamine or its analogues directly to food, food ingredients, animal feed, feed ingredients, or pelletizing agents" (Hilts and Pelletier, 2009). Individuals intentionally adulterate feed and food with melamine because of melamine's high nitrogen content, 66% by mass (Yang et al., 2009). This high nitrogen content gives an invalid estimate of protein content when the Kjeldahl method is used for protein analysis (Yang et al., 2009). Thus, feedstuff contaminated with melamine appears to have a crude protein (CP) content that is not representative of the amino acid content of the feedstuff (WHO, 2008a).

Cyanuric acid, along with ammeline and ammelide, are structural analogues of melamine (Tyan et al., 2009; WHO, 2008a) and all belong to the class of chemicals known as *s-triazines* (Wackett et al., 2002) (Figure 2.1.). Hydrolysis or amination of one *s-triazine* can result in the production of another *s-triazine*; the pathway through which

this occurs is depicted in Figure 2.2 (Baynes and Riviere, 2010). Jutzi et al. (1982) showed that degradation of melamine can occur by bacterial degradation. Filigenzi et al. (2007) stated that there is no known metabolism of melamine in mammals. However, Baynes and Riviere (2010) suggest that bacterial degradation might occur in the gastrointestinal tract or biological fluids.

Cyanuric acid is a crystalline powder (MSDS, 2010) with many common uses including its use in swimming pools (WHO, 2008b). Sodium dichloroisocyanurate is commonly used in swimming pools to disinfect water, and as it dissolves several compounds can be released including isocyanuric acid (Hilts and Pelletier, 2009). Cyanuric acid is not approved by the United States Food and Drug Administration (USFDA) for use as a non-protein nitrogen source in poultry or swine feed, but is approved for use in ruminant feed as a component of feed-grade biuret (Hilts and Pelletier, 2009; WHO, 2008b). Human exposure to cyanuric acid can occur by swallowing pool water, consuming contaminated drinking water, or consumption of fish which can accumulate the chemical (WHO, 2008b).

There are several possible routes by which a feedstuff can be contaminated by both melamine and cyanuric acid or a related compound. The first is through the intentional adulteration of a feedstuff with both compounds (WHO, 2008a). Another route is through bacterial degradation of melamine to cyanuric acid or other structurally related compounds (Jutzi et al., 1982). Degradation can also occur after or during production of melamine, resulting in a powder that is contaminated with melamine and other related analogs (Dobson et al., 2008). Therefore, there exist several possible routes

by which both melamine and related compounds can be found in biological systems simultaneously.

Toxicity:

Melamine, when individually consumed, has a low acute toxicity in mammals (FDA, 2009; WHO, 2008b). Melnick et al. (1984) determined the LD_{50} of melamine in rats to be 3,161 mg/kg body weight (BW). During the Melnick et al. (1984) study, an increase of bladder stones and hyperplasia was observed in rats fed 4500 ppm of melamine for 13 weeks. During a chronic 105 week study, Melnick et al. (1984) observed chronic inflammation in the kidneys of rats fed melamine. However, no toxic effects were observed in research conducted on dogs, cats, or swine by Lipschitz and Stockey (1945), Puschner et al. (2007), or Stine et al. (2011), respectively.

Additional reports have suggested that melamine can combine with uric acid to form insoluble compounds (Grases et al., 2009; Ogasawara et al., 1995). Grases et al. (2009) examined a calculus removed from the bladder of an 11-month-old girl that consumed baby formula contaminated with melamine. The calculus exhibited Fourier transform infrared (FTIR) spectra and scanning electron microscopy features that were identical to those of a melamine-uric acid crystal that was prepared in vitro (Grases et al., 2009). Further in vitro testing by Grases et al. (2009) revealed that at pH values less than 5.0, melamine and uric acid form insoluble compounds, "probably with a structure similar to that of the insoluble compound formed between melamine and cyanuric acid." A report published by the World Health Organization (WHO) (WHO, 2008a) stated that preliminary research by the USFDA showed that chickens, which excrete large amounts

of uric acid, form spherulites that are presumably composed of melamine-urate crystals that can dissolve quickly in formalin.

With the exceptions of humans, some primates, birds, and most vertebrates produce an enzyme, urate oxidase, which catabolizes uric acid to allantoin (Wu et al., 1989). Absence of this enzyme in humans causes serum uric acid levels to be 10 to 20 times higher compared to other mammals that express the enzyme (WHO, 2008a). Therefore, doses of melamine needed to produce melamine-uric acid stones in rats and other experimental animals may be higher than what is needed in humans (WHO, 2008a). Johnson et al. (1969) showed that rats with impaired uric acid oxidase have a higher probability of crystal formation in uric acid nephropathy. Additionally, a report in Pediatric Environmental Health Specialty Units (PEHSU) (PEHSU, 2009) stated that infants have lower glomerular filtration rates as compared to older children, possibly resulting in lower flow rate of urine through the tubules (PEHSU, 2009). Lower flow rate could possibly allow co-crystallization to occur between melamine and uric acid. PEHSU (2009) also reported that infants excrete uric acid into their urine at a greater rate than older children. This is supported by unpublished data from China that showed renal stones from infants consuming melamine contaminated formula to be comprised of melamine and uric acid in a 1.2:1 to 2.1:1 molar ration with no cyanuric acid present (WHO, 2008a).

Cyanuric acid, like melamine, has a low acute toxicity in mammals (OECD, 1999). Several experiments have shown that ingestion of cyanuric acid can cause renal damage, such as necrosis of the tubular epithelium, increased basophilic tubules, mineralization, and dilatation of the renal tubules (OECD, 1999). However, the LD_{50} for

cyanuric acid in rats is relatively high at 7,700 mg/kg BW (OECD, 1999). The no-observed-adverse-effect-level (NOAEL) for cyanuric acid was determined to be 150 mg/kg/day for male and female rats (OECD, 1999).

The high LD_{50}'s for melamine and cyanuric acid, when consumed alone, suggest that separately they are relatively nontoxic (Gonzalez et al., 2009). However, recent studies and case reports have demonstrated that the simultaneous consumption of both compounds is toxic to mammals even at low doses (Nilubol et al., 2009; Puschner et al., 2007; Reimschuessel et al., 2008). Stine et al. (2011) determined the NOAEL for pigs fed melamine and cyanuric acid in combination for 28 days to be 1.0 mg/kg BW/day, or approximately 25 mg/kg of each compound in the diet. However, research conducted by Brand (2011), suggested that in young birds the combination of melamine and cyanuric acid is less toxic than in companion animals. Brand (2011) stated that the addition of cyanuric acid to melamine contaminated diets was able to alleviate the negative effects that were observed in poults that consumed melamine alone.

Increased toxicity from consumption of both melamine and cyanuric acid comes from the ability of melamine and cyanuric acid to self-assemble and form a hydrogen-bonded bimolecular network (Perdigao et al., 2006). Self-assembley can lead to the formation of insoluble compounds that can precipitate in the kidneys, leading to renal failure (Seffernick et al., 2010). Dobson et al. (2008) observed that melamine cyanuric acid crystals were present in contaminated wheat gluten and were not disrupted by processing. Tolleson (2009) indicated that lower pH levels, such as those present in the digestive tract, increases the solubility of melamine and cyanuric acid. This enhanced solubility facilitates increased absorption from the gastrointestinal tract (Tolleson, 2009).

Due to the different pKa's of the compounds, 6.9 for cyanuric acid and 5.0 for melamine, it is probable that the acid is absorbed in the stomach while the base is absorbed in the small intestine (Dobson et al., 2008). Different sites of absorption would explain why the two compounds do not recombine after leaving the stomach and entering an environment with a higher pH (Dobson et al., 2008).

After absorption, the compounds are evenly distributed throughout total body water (Hammond et al., 1986; Lipschitz and Stockey, 1945), but only precipitate out in the kidneys (Dobson et al., 2008). Dobson et al. (2008) proposed several explanations as to why crystal formation only occurs in the kidney: 1) recombination does not occur until concentrations of the compounds exceeds a critical point, which could occur as the compounds move down the osmotic gradient in the kidneys; 2) melamine and cyanuric acid interfere with uric acid metabolism, which would precipitate in the tubules and become a substrate for melamine and cyanuric acid to precipitate.

Tolleson (2009) reported that renal calculi were commonly detected in the distal tubles, collecting ducts of pets, or the entire nephron in laboratory animals fed 400 mg/kg of melamine plus cyanuric acid. Tolleson (2009) went on to suggest several different pathways for renal failure to occur in association with triazine nephrotoxicity. The first is classified as post-renal, and relates to urinary obstruction downstream from the glomerulus that interfers with renal output. The second is classified as pre-renal, and involves restriction of afferent blood flow to the glomeruli by decreaed cardiac output, decreased arterial blood pressure, hypovolemia, thromboembolism, or arteriosclerosis. Finally, Tolleson (2009) stated the nephrotoxicity could be associated with renal damage,

in which kidney cells are damaged by local ischemia, oxidative stress, or direct cytotocixity leading to cellular death.

Background Issue:

Pet Food:

In March of 2007, deaths related to renal failure were documented in dogs and cats across North America and South Africa (Hilts and Pelletier, 2009; WHO, 2008a). Investigation into the deaths revealed that pet food consumed by affected animals was contaminated with melamine and related analogs (Byungghul et al., 2008). It was later reported that wheat gluten imported from China and incorporated into the pet food was contaminated with melamine and other similar compounds (AVMA, 2007; Brown et al., 2007; Burns, 2007). The intentional contamination of wheat gluten with melamine was done in an attempt to artificially increase the crude protein content of wheat gluten, allowing it to pass for a more valuable feedstuff (Hilts and Pelletier, 2009). Analysis of some 200 pet food samples revealed melamine concentrations ranging from 0 to 2,263 mg/kg (Cianciolo et al., 2008). Cyanuric acid was also found in many of the pet foods at levels greater than 10 mg/kg (Hilts and Pelletier, 2009). The USFDA estimated that contaminated wheat gluten contained between 0.2 and 9 % melamine (Hilts and Pelletier, 2009).

By April of 2007 some 100 different brands of dog and cat food had been voluntarily recalled across the United States (FDA, 2007). Approximately four months later the American Association of Veterinary Laboratory Diagnosticians (AAVLD) reported that 347 cases met the criteria for "pet food-induced nephrotoxicity" based on

high concentrations of blood urea nitrogen (BUN) and creatinine (Burns, 2007). Of these reported cases, 235 involved cats and 112 involved dogs, with mortality occurring in 61 and 74 % of cats and dogs, respectively (Burns, 2007). It was later discovered that deaths in 2007 were not the first associated with melamine toxicity (Bhalla et al., 2009). In 2004, pet deaths occurring in Asia related to food consumption was attributed to mycotoxicosis. However, reevaluation of necropsies showed identical clinical, histological, and toxicology findings as the pets involved in the 2007 melamine incident. These similar findings suggest that the 2007 incident was not the first time feed had been contaminated with melamine (Brown et al., 2007).

Production Animal Feed:

Following the contamination of pet food, it was determined that waste material containing melamine and/or cyanuric acid from pet food manufacturing was incorporated into swine, poultry, (Buur et al., 2008; Karbiwnyk et al., 2009; USDA, 2007a) and aquaculture feed (Karbiwnyk et al., 2009). When pet food scraps are used in swine diets it usually comprises 5 to 10 % of the diets (Hilts and Pelletier, 2009). However, it was found that some hogs were fed feed that contained 50 to 100% pet food scraps (Hilts and Pelletier, 2009). Bakery meal usually contains pet food scraps and can be included in poultry rations between 3 and 15% (Hilts and Pelletier, 2009). Testing by the United States Department of Agriculture (USDA) and other private labs revealed melamine and related analogs in 56 samples of pet food scraps, 27 samples of bakery meal, 17 samples of swine feed, 21 samples of poultry feed, and 7 samples of fish feed (Hilts and Pelletier, 2009). With the limit of detection (LOD) set at 50 parts per billion (ppb), melamine was

found in concentrations up to 1,952, 59.6, 120, and 400 mg/kg in pet food scraps, bakery meal, swine feed, and fish fed, respectively (Hilts and Pelletier, 2009). Cyanuric acid was found at levels as high as 2,180, 146.3, 22.2, and 2.63 mg/kg in pet food scraps, bakery meal, swine feed and poultry feed, respectively (Hilts and Pelletier, 2009).

After it was confirmed that contaminated feed was fed to swine and poultry, the USDA Food Safety and Inspection Service (FSIS) stated "that risk to human health from consuming pork or poultry products from these animals was likely to be very low" (USDA, 2007a). However, the FSIS could not be sure that products from swine and poultry that had consumed contaminated feed was not itself contaminated (USDA, 2007a). Therefore, in cooperation with state and local producers, all animals that had consumed feed contaminated with melamine or related compounds were placed under quarantine (USDA, 2007a).

For example, on April 18th, 2007, a 1,500 head swine farm in California was placed under quarantine when urine from swine tested positive for melamine (CDFA, 2007). During the time of quarantine the FSIS analyzed urine and muscle tissues from animals that had consumed contaminated feed. Analysis of these samples allowed for a relationship to be drawn between the level of melamine in the feed and levels in animal tissues. Melamine was found below the LOD of 50 ppb in all poultry and swine tissues tested, even in pigs that had consumed diets that contained pet food scraps at levels between 50 and 100% (Hilts and Pelletier, 2009). The highest level of melamine in swine urine was 2,220 mg/kg and the levels decreased as time from exposure to melamine increased (Hilts and Pelletier, 2009). On May 15th, 2007, after analyzing tissue samples, the USDA FSIS stated "that there is very low risk of harm to humans from

eating food containing low levels of melamine or related compounds" (USDA, 2007b). The FSIS explained that in a worst case scenario if all the food a person ate in one day contained melamine and cyanuric acid at levels potentially present in the meat, the possible exposure would be about 250 times lower than the dose considered safe (USDA, 2007b). On the same day, the USDA announced that poultry and swine products from animals that had consumed contaminated feed were "not adulterated" and were able to be offered for slaughter (USDA, 2007a).

Human Food:

In September 2008, China reported that some 52,875 children had been treated for renal complications, after having consumed infant formula and other related dairy products that were contaminated with melamine (Chan et al., 2008; WHO, 2008a). By November of 2008 some 294,000 infants had been affected, with more than 50,000 hospitalized and six reported deaths (WHO, 2008a). It was determined that melamine was used to artificially increase the crude protein of milk that was used in the production of infant formula (PEHSU, 2009; WHO, 2008a). This contamination raised concerns across the United States, since formula manufactured in Asia was available for sale in the U.S. (PEHSU, 2009).

A 2009 publication for pediatric health professionals (PEHSU, 2009), stated that infants are more vulnerable to melamine induced renal failure due to several factors. One being that formula is the primary food source of most infants, as compared to older children who will eat a variety of food, thus receiving less melamine per unit of body weight than infants. Infants also have smaller lumens in their urinary tract, which can

more easily become irritated by urinary stones. Finally, infants have a lower glomerular filtration rate and higher levels of uric acid in their urine (PEHSU, 2009). Ogasawara et al. (1995) and PEHSU (2009) suggested that uric acid might form crystals with melamine in infants.

During the 2008 China incident documented clinical signs of melamine toxicity included irritability, vomiting, fever, hematuria, dysuria, oliguria, anuria, high blood pressure, oedema, and pain in the kidney areas (WHO, 2008a). Multiple stones were reported and typically occurred without clinical signs. An eight-month-old female who had consumed contaminated infant formula for 15 days presented with multiple stones in both kidneys (WHO, 2008a). Unpublished data released by the Chinese showed that these renal stones were composed of melamine and uric acid in a 1.2:1 to 2.1:1 molar ratio, with no cyanuric acid present in the stones (WHO, 2008a). Serum potassium was 5.57 mmol/l, blood urea nitrogen was 24.7 mmol/l, and creatinine was 575.9 μm/l in affected infants. After five days of peritoneal dialysis and intravenous sodium bicarbonate the stones passed and the infant recovered. It is believed that most of the melamine related deaths in humans was due to a lack or delay of treatment (WHO, 2008a) .

In 2007, the WHO established a tolerable daily intake (TDI) for melamine at 0.2 mg/kg BW, which applies to the whole population, including infants (WHO, 2007). In the same publication, the WHO also established a TDI for cyanuric acid at 1.54 mg/kg BW (WHO, 2007). Analysis of the adulterated infant formula in China revealed that melamine, cyanuric acid, ammeline, and ammelide, were present in the raw material used to manufacture the infant formula at levels of 188,000, 3.2, 14.9, and 293.0 mg/kg,

respectively (WHO, 2008a). Dietary exposure to the median level of melamine that was reported in the most contaminated brand of infant formula was between 8.6 and 23.4 mg/kg BW (WHO, 2008a). This is approximately 40 to 120 times the TDI set by WHO (WHO, 2008a). After this, many countries established limits for melamine in infant formula and other foods (WHO, 2008a). In 2008 the FDA set a TDI at 0.63 mg/kg BW/day (FDA, 2009) before reestablishing it to 0.063 mg/kg BW/day (FDA, 2008). The limit established for infant formula was 1 mg/kg, while 2.5 mg/kg was used as the limit in other foods (WHO, 2008a). "These limits provided a sufficient margin of safety for dietary exposure relative to the TDI" (WHO, 2008a).

After the 2008 adulteration of milk products, including infant formula, reports were published raising concerns that other foods originating from China might be contaminated with melamine. Foods included fruits, vegetables, fresh eggs, powdered and liquid egg products, non-dairy creamers, ammonium bicarbonate, and animal feed (FSANZ, 2008; HKCFS, 2008; WHO, 2008a). This resulted in testing of these types of food products and products containing these ingredients (WHO, 2008a).

Analysis:

Several different methods are available for the detection and quantification of melamine and its related analogs (WHO, 2008a). Methods discussed in a report released by the WHO (2008a) include enzyme-linked immunosorbent assay (ELISA), high-performance liquid chromatography (HPLC) – ultraviolet (UV)/diode array detection (DAD), gas chromatography (GC) –mass spectrometry (MS), GC-MS/MS, and liquid chromatography (LQ) –MS/MS. Several other methods have been developed or improved

in the years following and include surface-enhanced Raman spectroscopy (SERS), isotope dilution, and SERS coupled with gold nanosubstrates (Tyan et al., 2009). Each method has its own level of selectivity, sensitivity, and cost associated with it (WHO, 2008a). Some methods are only suitable for the detection of melamine, while others can detect multiple compounds simultaneously (WHO, 2008a). A few techniques are only appropriate for detecting melamine and should not be used for quantifying melamine, while others can be used for both detecting and quantifying melamine in a particular substrate (WHO, 2008a).

Before detection and or quantification of melamine or a related analogue can occur sample extraction must first be performed (WHO, 2008a). During extraction care must be taken to avoid hydrolysis of melamine and its analogues to cyanuric acid, especially when using extremely basic extraction conditions (WHO, 2008a). It is also critical to dissociate melamine from relatively insoluble complexes with cyanuric acid before analysis (WHO, 2008a). Therefore, the pH of the extraction solvent may need to be adjusted to cause melamine to dissociate from insoluble complexes (WHO, 2008a) . Commonly an acidic aqueous solvent mixture is used to extract samples followed by mixed-mode solid-phase extraction, when sensitive detection methods will be used (WHO, 2008a). For melamine analysis cation exchange/reverse phases can be used (Andersen et al., 2008). Smoker and Krynitsky (2008) isolated cyanuric acid using mixed-mode anion exchange sorbents. Other solid phase sorbents used to aid in the extraction include graphitized carbon phases and C_{18} (Chou et al., 2003; WHO, 2008a).

ELISA kits have been developed and considered for the detection of melamine and other *s-triazines* when high-throughtput screening of samples is needed (Garber,

2008). Depending on the matrix being analyzed and the extraction method used, quantification limits for melamine detection are between 0.1 and 25 mg/kg (WHO, 2008a). WHO (2008a) stated the ELISA test should be used for screening purposes only and that positive results should be confirmed using a more selective confirmatory method. Tyan et al. (2009) also stated that if samples test positive for melamine then other tests may be required to ensure that cross-reacting compounds did not create a false positive and to determine the exact level of melamine.

The most common methods used to detect melamine in foods and feedstuff are GC-MS and HPLC combined with UV or MS detectors (Tyan et al., 2009). LOD and linear ranges for the calibration curves for these GC-MS and HPLC-UV/MS are 0.1 to 0.02 ppm and 0.01 to 5 ppm, respectively (Tyan et al., 2009). The WHO (2008a) reported that quantification limits for melamine and its related analogs using HPLC-UV/DAD range from 0.05 to 65 mg/kg. The WHO (2008a) also stated that HPLC-UV/DAD can be used as a confirmatory method only if it has been validated thoroughly for the matrices of interest.

GC-MS and LC-MS are used by the US FDA to identify melamine in foods (Tyan et al., 2009). These methods, along with tandem mass spectrometry (MS/MS) based methods, can be used as either a screening or confirmatory method due to their medium to high level sensitivity (WHO, 2008a). For GC-MS and GC-MS/MS quantification, limits range from 0.05 to 10 mg/kg and 0.002 to 5 mg/kg, respectively (WHO, 2008a). A method for the analysis of porcine muscle tissue by solid-phase extraction, followed by HPLC-MS/MS, is described in a report by (Filigenzi et al., 2007). Chou et al. (2003) described a method for determining levels of cyromazine and melamine in poultry meat

and eggs by HPLC analysis. The USFDA has also published several methods for melamine analysis (Andersen et al., 2007; Smoker and Krynitsky, 2008; Turnipseed et al., 2008).

A new approach to measure both melamine and cyanuric acid is by surfaced-enhanced Raman spectroscopy (SERS) coupled with gold and other various nanosubstrates (Mermelstein, 2009) . SERS is a branch of Raman spectroscopy that measures the molecular vibrations made by scattering light (He et al., 2008). He et al. (2008) demonstrated that SERS could provide a fast method for detection of melamine and its derivatives in aqueous solutions. SERS is able to rapidly detect 2 ppm of melamine in milk (Mermelstein, 2009), but has the potential of sample detection at the parts per billion (ppb) level or even a signal molecule (He et al., 2008). A major advantage of SERS is that melamine could be detected on site and in real time, about 15 minutes per sample (He et al., 2008; Liu et al., 2010; Mermelstein, 2009).

Biological Effects:

Case studies and experiments:

Humans:

Following the 2008 outbreak of melamine induced renal failure in children several case reports were published in a report by the WHO (2008a). The reports documented clinical symptoms of infants to include crying, vomiting, fever, hematuria, dysuria, oliguria, anuria, oedema, high blood pressure, and pain in the kidney area. However, it should be noted that most children did not show clinical signs. After screening 1,129 children in Taiwan, Ho et al. (2009), suggest that hypercalciuria,

hematuria, or positive abdominal radiographs were not significantly increased in patients that presented with kidney stones. Therefore, these systems should not be used to diagnose renal damage associated with melamine, instead ultrasonography should be performed to aid in diagnoses (Ho et al., 2009).

It was determined that renal stones associated with the 2008 China incident contained uric acid and melamine with no cyanuric acid detected (WHO, 2008a). Grases et al. (2009) suggested that such calculus could be prevented by the alkalinization of urine. Grases et al. (2009) suggestion was based on in vitro testing where melamine and uric acid only formed crystals when the pH of the aqueous solution was lower than 5.0. The WHO (2008a) reported that children in China were treated with intravenous sodium chloride with dextrose and sodium bicarbonate or sodium citrate to increase urine pH to 6.5 to 7.0. At the same time, children that presented with renal failure or anuria were placed on dialysis and/or surgical removal of the stones was performed.

Dogs and Cats:

Some of the earliest animal research investigating melamine was done by Lipschitz and Stockey (1945) who demonstrated the diuretic effects of melamine in dogs. Results of Lipschitz and Stockey (1945) showed that melamine increased water output as well as NaCl ouput, in a positive dose related manor. The research by Lipschitz and Stockey (1945) also showed that 60 to 86.5 % of the melamine fed to dogs could be recovered in the urine within 24 h of administration. Additional research involving the effect of melamine on dogs and cats was not conducted until the reports of melamine associated renal failure occurred in 2007.

Brown et al. (2007) examined tissues and the medical history of ten cats and six dogs that died during 2007 and were reported to have consumed contaminated pet food. All animals had exhibited anorexia, vomiting, lethargy, polyuria, and polydipsia. Fourteen animals had blood serum analysis reported with creatinine levels from 7 to 15 mg/dl (reference range 0.9 to 2.1 mg/dl) and BUN greater than 130 mg/dl (reference range 20 to 34 mg/dl) (Brown et al., 2007). BUN and creatinine levels are used as indicators of kidney function,with elevated levels indicating kidney failure (Excyclopedia, 2011). Eight of the 16 animals in the Brown et al. (2007) study had extrarenal lesions associated with uremia. All 16 animals had polarizable crystals in the distal tubules and collecting ducts of the kidney.

Cianciolo et al. (2008), exposed 70 cats to commercial canned or pouched contaminated pet food and were able to document the histological and clinical signs associated with melamine and cyanuric acid toxicity. In the study, 43 of the cats developed signs of toxicosis. Clinical signs ranged from inappetence with or without vomiting, polydipsia, polyuria, dehydration, lethargy, and anorexia. Gross examination of 14 cats revealed 10 with bilateral enlargement of the kidneys. Further histological evaluation of the kidneys showed aggregates of gold-brown crystals in the distal segments of the nephron. Most crystals were between 15 and 30 μM in diameter, with two concentric rings, giving the appearance of spokes radiating from the center (Cianciolo et al., 2008). Puschner et al. (2007) also documented crystals as being pale translucent, yellow to clear, with morphology that ranged from fan-shaped to starburst prism to globular in cats fed combinations of melamine and cyanuric acid. All the

crystals observed in the Puschner et al. (2007) study displayed multicolored birefringence to cross-polarized light.

Feeding trials conducted by Puschner et al. (2007) in which cats received 0.5 and 1.0% melamine in their diet, showed no evidence of renal failure after 11 days on test. In the same study, a cat receiving cyanuric acid up to 1.0% of its diet, showed no evidence of renal failure as measured by serum creatinine and urea nitrogen. However, cats fed a combination of melamine and cyanuric acid in a one-to-one ratio at levels of 0.2, 0.5, and 1.0% of their diet, showed slight depression, vomiting and anorexia after 12 hours on test. Cats fed melamine in combination with cyanuric acid showed histological lesions limited to the kidneys with crystals present in the lumina of the collecting ducts and within the distal tubules. Puschner et al. (2007) demonstrated that a single oral exposure of 32 mg/kg BW of both melamine and cyanuric acid to cats can result in acute renal failure.

Rodents:

Rats and mice have been used in several studies to determine the effects of melamine and cyanuric acid on animals (Dobson et al., 2008; Mast et al., 1983; Melnick et al., 1984; Ogasawara et al., 1995). An early study conducted by Mast et al. (1983) showed that melamine is not metabolized in male rats after a single oral dose of approximately 1.3 mg/kg BW. Mast et al. (1983) also stated that melamine appears to be distributed in total body water with most excreted via the urine. Jingbin et al. (2010) were able to show that melamine consumed by a pregnant rat can be passed through the placenta to the fetus in a dose dependent manner.

Melnick et al. (1984) conducted three experiments to study the effects of acute, (14 days), subchronic (13 weeks), and chronic (103 weeks), exposure to melamine in male and female mice and rats. The LD_{50} of melamine in male rats and mice was 3,161 and 3,296 mg/kg BW, respectively. While the LD_{50} for female rats and mice was reported as 3,828 and 7,014 mg/kg BW, respectively. Melnick et al. (1984) also noted that the urinary bladder was the only organ affected after exposure to melamine for 13 weeks. Only chronic exposure to melamine at levels higher than 4,500 ppm caused a significant increase in kidney inflammation. An increase in bladder stones and hyperplasia of the transitional epithelium of the urinary bladder was reported in male rats that receive melamine for 13 weeks. Data showed that male rats and mice fed melamine had a greater probability of developing urinary bladder stones than females (Melnick et al., 1984).

Research conducted shortly after the sudden deaths of dogs and cats in 2007 by Dobson et al. (2008) found that feeding rats 10, 30, or 100 mg/kg of ammelmine or ammelide alone did not cause kidney weights, BUN, or serum creatinine to increase. Dobson et al. (2008) went on to study the effects of a mixture of trizaine in rats by feeding 400 mg/kg BW melamine and 40 mg/kg BW each of ammamine, ammeline, and cyanuric acid. A mixture of 400 mg/kg BW melamine and 400 mg/kg BW cyanuric acid was also fed to rats. Both mixtures resulted in toxicity, with diuresis as the initial symptom in most animals. By day three, animals (especially in the melamine and cyanuric acid group), were oliguric with some hematuria. Serum analysis also showed increased levels of BUN, creatinine, and creatinine clearance, which are indicators of

impaired renal function. Kidney weights were higher in rats fed a mixture of triazines, with the tubules streaked with brownish-yellowish precipitates (Dobson et al., 2008).

Fish:

It has been documented that food used to feed farm-raised fish was also contaminated with melamine (Tolleson, 2009; WHO, 2008a). However, little research has been conducted on the effects of melamine on fish. Reimschuessel et al. (2008) fed melamine and cyanuric acid alone or in combination to tilapia, channel catfish, rainbow trout, and Atlantic salmon, for 3 days. Targeted doses were 400 mg/kg of melamine or cyanuric acid alone, or a combination of 400 mg/kg of each compound. Two salmon that received the combination diet died early in the study and were replaced with salmon fed 200 mg/kg of melamine and cyanuric acid (Reimschuessel et al., 2008).

Results of the Reimschuessel et al. (2008) study documented that trout, salmon, and some catfish fed the combination diet passed white feces, with all combination fed fish presenting with similar material in the intestinal lumen. No crystals were detected in fish fed diets that contained melamine alone. Twenty five of the 26 kidneys from fish fed combinations diets contained many crystals arranged in radial spherulities within the tubules. These crystals were described as gold-brown and needle-like and had formed radial spheroid aggregates. Some kidney tubules were dilated, contained necrotic cells, and had basophilic, regenerative epithelium along the basement membrane (Reimschuessel et al., 2008). Analysis of tissue samples revealed the presence of melamine and cyanuric acid one day after administration of the compounds started. Results also indicated that muscle from catfish fed a combination diet for one day had

lower melamine and cyanuric acid concentrations than catfish fed melamine or cyanuric acid alone. Lower residue levels in combination fed fish can be attributed to precipitation of the melamine cyanurate complex in the gastrointestinal tract and kidneys of fish (Reimschuessel et al., 2008).

Ruminants:

Most research done in ruminants involves investigating the use of melamine as a non-protein nitrogen (NPN) source (Clark, 1966; Newton and Utley, 1978). However, after the melamine contamination of feed and food in 2007 and 2008, Shen et al. (2010) reported on the ability of melamine to be transferred from feed ingredients to milk in dairy cattle. Shen et al. (2010) fed 0, 90, 270, and 450 mg of melamine to Holstein cows for 13 days and found milk from cattle receiving high dietary melamine levels to contain more melamine than milk from cattle fed lower levels of melamine. It was also reported that transfer efficiency was not associated with dietary melamine level, but instead correlated to milking ability of cows, with higher producing cows able to transfer more melamine from the diet to the milk. Using equations produced from data analysis and maximum melamine levels for food ingredients, set by the USFDA, Shen et al. (2010) suggested that if the mean daily melamine intake of a dairy cow exceeds 312.7 mg the milk should not be used to produce infant formula. Shen et al. (2010) also noted that if intake exceeds 715.1 mg the milk should not be used to produce common milk powder because the powder might exceed levels set by the FDA. However, Shen et al. (2010) went on to state that more testing is needed to confirm these levels.

Newton and Utley (1978) included either cottonseed meal, urea, or melamine at 0.5% of steer diets in order to evaluate melamine as a NPN source. After three days on test, the mean ruminal ammonia concentration was lower for both melamine and cottonseed meal, at 3.6 and 8.4 mM, respectively. It has been reported that 3.57 mM is needed for maximum rumen protein synthesis, suggesting that melamine at the level fed did not provide enough ammonia for maximum microbial protein synthesis (Newton and Utley, 1978). It was also reported that while melamine was digested to the same extent as cottonseed meal, more nitrogen appeared in the urine of melamine fed steers. Newton and Utley (1978) went on to state that under test conditions used in their research melamine did not provide adequate nitrogen supplementation for cattle and made no mention of renal effects.

Clark (1966) reported feeding sheep melamine in multiple or single doses that ranged from 10 to 100 mg. Administering 10 mg of melamine per day had toxic effects in one sheep after 16 consecutive days of dosing and after 31 days for another sheep. A third sheep went unaffected for 39 days, before it was removed from the trial. Sheep fed melamine that underwent autopsy showed multiple crystals in the kidney tubules, with nephrosis and erosive abomasitis also present. Clark (1966) stated that death occurring after melamine administration was due to blockage of the kidney tubules with crystals resulting in anuria and uraemia.

Poultry:

While melamine appears to be relatively non-toxic in most mammals including rodents, pigs, and fish (Melnick et al., 1984; Reimschuessel et al., 2008; Stine et al.,

2011), studies by Brand (2011) and Bermudez et al. (2008) suggest that melamine can be toxic in poultry species. Also, in contrast to findings that combinations of melamine and cyanuric acid are toxic to pigs, fish, and rodents (Dobson et al., 2008; Reimschuessel et al., 2008; Stine et al., 2011), research by Brand (2011) suggest that melamine fed in combination with cyanuric acid is not as toxic in turkeys.

Brand (2011) included melamine at levels up to 3.0% of a broiler diet and observed that melamine ≥ 1.0% of the diet is toxic, but mortality was not significantly increased when fed at levels less than or equal to 2.0%. An increase in relative kidney and liver weights was noted in broilers fed melamine greater than or equal to 1.5 and 2.5% of the diet, respectively. However, residue levels from broilers fed greater than or equal to 1.0% melamine may exceed what is considered safe by the FDA. Brand (2011) went on to report that melamine present in the bile of broilers suggests that avian species can clear melamine through their bile. Ding et al. (2011) reported that broilers fed 100 mg/kg melamine had increased glutamic-pyruvic transaminase (GPT) and uric acid levels after 21 days on test. After 32 days on test, broilers fed 100 mg/kg showed similar growth performance as controls but showed toxicity in the liver and kidney (Ding et al., 2011).

Poults, like broilers, can tolerate up to 5,000 mg/kg of melamine with no adverse effect on growth performance (Brand, 2011). However, at this level the kidneys of poults do contain significant concentrations of melamine. Moderate to severe tubulointerstitial nephritis with mineralized casts were observed in the collecting tubules and collecting ducts of poults fed melamine. Brand (2011) also reported 32% mortality in poults fed greater than or equal to 1.5% melamine in the first ten days of feeding, with poults

surviving past day 10 appearing to be less sensitive to melamine. A similar occurrence was reported by Lu et al. (2009), who reported lower melamine residue levels in broiler that consumed contaminated diets for 42 d as compared to broilers that consumed contaminated diets for 28 days.

Brand (2011) also evaluated the toxicity of cyanuric acid alone in broilers and poults when it is included at up to 3.0% of their diets. Mortality, feed intake, body weight gain, and feed conversion of broilers were not affected by cyanuric acid. This suggests that cyanuric acid up to 3.0% of a diet is not toxic to broilers. Cyanuric acid did not affect mortality, body weight gain, or feed conversion in poults. Gross examination of poults showed no toxic effects with kidneys appearing unremarkable. These data suggest that cyanuric acid fed alone, up to 3.0% of the diet, is not toxic to young broilers and poults (Brand, 2011).

Feeding a combination of melamine and cyanuric acid, in a one-to-one ratio up to 3.0% of a broilers diets, Brand (2011) observed no effect on mortality. However, feeding a combination of melamine and cyanuric acid did cause a depression in feed intake, body weight gain, and an increase in relative kidney and liver weights. Crystals documented in kidneys of broilers fed 0.5, 1.0, and 1.5% combination diets had the characteristic polarizable melamine-cyanuric acid crystals describe in the literature. The polarizable nature of melamine-cyanuric acid crystals can be used to differentiate them from melamine crystals (Brand, 2011).

In poults, the addition of cyanuric acid to a diet that contained melamine, appeared to reduce the negative effects of melamine on feed intake and body weight gain (Brand, 2011). This same effect was observed on the relative kidney weight of poults fed

the combination diet. However, BUN levels were elevated in poults fed combination diets, but all values fell within or near normal values for poultry species. Brand (2011) stated that the addition of cyanuric acid to melamine contaminated diets was able to prevent the formation of kidney lesions caused by melamine in young turkeys.

Two experiments have been conducted to study the effects of melamine in ducks. The first conducted by Yan et al. (2009) involved feeding melamine at levels ranging from zero to 1,000 mg/kg. Feeding less than 50 mg/kg melamine to ducks for 42 days did not result in a detectable amount of melamine residue in the breast, liver, or kidney tissue. However, melamine residues in tissue increased linearly with increasing dietary levels greater than 100 mg/kg. It was also noted that concentrations of 500 and 1,000 mg/kg of melamine caused an uneven distribution of melamine to be deposited in the tissues, with the kidneys having the highest concentration followed by liver and breast (Yan et al., 2009)

Gao et al. (2010) conducted research to determine the effects of graded levels of melamine, up to 100 mg/kg, in laying ducks. Gao et al. (2010) reported no effect on average egg weight, egg production, feed intake, or feed conversion in Jinding laying ducks fed less than or equal to 100 mg of melamine per kg of diet. Ducks fed melamine at levels between 50 and 100 mg/kg had increased BUN levels. Histological lesions were also reported in the kidneys of ducks fed greater than or equal to 25 mg/kg melamine. Tubular cell necrosis and lymphocytic infiltration of the kidney was noted. Gao et al. (2010) also noted that dietary melamine can be transferred to eggs, with melamine concentrations in eggs increasing as dietary melamine levels increase.

Swine:

Shortly after the initial reports of melamine toxicity in 2007, pharmacokinetic research of melamine in the pig was conducted by Baynes et al. (2008). Intravenous injection of 6.13 mg of melamine/kg BW was administered to five weanling pigs and blood samples were collected over a 48 h period. This equates to approximately 120 mg melamine/kg feed, assuming 100 kg pigs consuming 3 kg of feed daily (Buur et al., 2008). Distribution of melamine in the pigs was close to total body water, suggesting distribution is limited to the extracellular fluid compartment and is not distributed to most organs (Baynes et al., 2008). Baynes et al. (2008) also found that melamine is rapidly removed from the pig via renal filtration, with a half life of 4.07 ± 0.39 hours. Therefore, Baynes et al. (2008) suggested a one-compartment, first order kinetics model with first order elimination.

Buur et al. (2008), utilizing physiologically based pharmacokinetic models, was able to estimate the time that swine should be withheld from slaughter after single and chronic oral exposures of 3 and 5.12 mg/kg BW of melamine. Two parameters used by Buur et al. (2008) in the models were; 1) swine with kidney residue levels below 50 ppb were considered safe for consumption; 2) due to the polar characteristics of melamine, hepatic clearance was considered insignificant. Physiologically based pharmacokinetic modeling estimate withholding swine from slaughter for 19.2 and 20.9 h for signal doses of 3.0 and 5.12 mg/kg BW respectively. For seven day, chronic exposure withholding times were estimated at 20 and 21.3 h for 3.0 and 5.12 mg/kg BW respectively.

Around the same time as the pharmacokinetic research was being conducted, several case studies involving the possible contamination of swine feed and resulting

morbidity and mortality of swine herds were reported. The first, by Nilubol et al. (2009), reported below average gain, slight pallor, and increased mortality at a swine farm in Thailand between February and May of 2007. It was later determined that the starter diet was contaminated with melamine, cyanuric acid, and ammeline at concentrations of 3,209, 1,126, and 949 mg/kg, respectively. Mortality started approximately two weeks after weaning at 21 days of age, and one to two months later approached 100% or 4,000 weaned pigs. Pathologic examination of 10 mortalities revealed five with kidneys that appeared yellowish, slightly swollen, glistening, and showed perirenal edema. The renal pelvis was dilated with crystalline precipitates evident on the surface that was cut. Histological lesions appeared in the cortex and medulla of the kidney and were also observed throughout the nephron, with more appearing in the distal section. Yellow-brown crystals with radiating striations caused epithelial disruption and necrosis with a moderate number of intratubular and peritubular neutrophils observed in the distal tubules and collecting ducts. Two pigs showed elevated levels of blood urea nitrogen at 121.2 and 157.9 mg/dl, and elevated creatinine levels at 11.9 and 15.0 mg/dl. Mortality was attributed to the physical presence of crystals formed by the interaction of melamine and cyanuric acid.

The second case study, reported by Gonzalez et al. (2009), involved between three to four hundred 45 to 60 day old piglets from five different farms in Spain. Clinicial signs started several days post weaning and included anorexia, depression, lethargy, and polydipsia. Necropsy was conducted on nine piglets, with gross abnormalities and histological lesions limited to the kidneys. The kidneys appeared moderately enlarged and firm with slightly irregular, orange, cortical surfaces with

numerous multifocal yellow foci measuring 0.5 mm in diameter extending from the cortex to the medulla. Crystals found in the cortex and medulla appeared multicolored and birefringence with cross-polarized light. Crystals ranging from 20 to 60 μm, were round and located in the lumen of distal convoluted tubules and collecting ducts. Appearance of crystals varied with some having "basophilic round centers surrounded by concentric lamellae with blue radial striations while others appeared pale green and irregular or striated aggregates" (Gonzalez et al., 2009). Infiltrates included macrophages, lymphocytes, plasma cells, and multinucleated foreign body-type cells and were associated with chronic inflammation. Feed samples were not available for analysis, but investigators believed that ammeline and ammelide were probably the major contaminants of the feed since concentrations of these two compounds were higher than concentrations of melamine and cyanuric acid in the kidney.

The final case study, reported by Lee et al. (2011) occurred in Malaysia during 2008. Yeast contaminated with 30,064 ppm melamine, was included in a pig diet at 5.0 %, giving the diet a melamine concentration of 750 to 1,500 ppm. Pigs consuming this diet appeared inappetant, anorexic, thin, dull, and depressed. The kidney was the only organ affected, appearing yellowish, discolored, and enlarged. A few kidneys from severely affected pigs were small in size with clear pitting and dimpling, dark red and brown in color, with acute tubular nephrosis and interstitual nephritis with multiple cysts present in the cortex.

To provide experimental data on melamine toxicity, Reimschuessel et al. (2008) feed 400 mg/kg BW of melamine and cyanuric acid alone or in combination to 16 week old Yorkshire-crossed pigs. In each treatment either melamine, cyanuric acid, or

melamine plus cyanuric acid was fed to one pig for a three day period. Pigs fed melamine or cyanuric acid alone showed no gross lesions or crystals present in the kidneys. Blood urea nitrogen and creatinine, both fell within normal ranges for pigs of the same age and sex. Kidneys from pigs fed the combination of melamine and cyanuric acid appeared flaccid, with small red foci, and were associated with a large amount of edema. The combination fed pig also had elevated blood urea nitrogen levels and creatinine levels, and crystals were detected in wet mounts of kidney tissue. Crystals appeared golden-brown and formed radial spheroid aggregates.

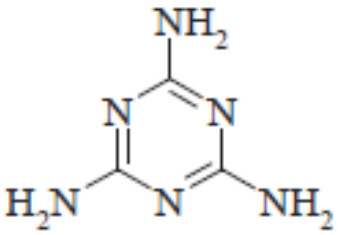
More recently, a NOAEL was determined by Stine et al. (2011). First, a preliminary seven day study was conducted in which 0, 1.0, 3.3, 10.0, 33.0, or 100 mg/kg BW/day of melamine-cyanuric acid or 200 mg/kg BW/day of melamine or cyanuric acid alone was fed to weanling barrows. Barrows fed 100 mg/kg BW/day of melamine-cyanuric acid had lower weight gains than controls and elevated blood urea nitrogen and creatinine levels. A dose related response for kidney and relative kidney weight was observed in barrows fed 10 mg/kg BW/day and higher of melamine-cyanuric acid combinations. Finally, crystalline structures were found in the medulla and cortex of kidneys from barrows fed 10 mg/kg BW/day and higher of melamine-cyanuric acid combinations, and barrows fed 200 mg/kg/day of melamine alone. No difference in urine analysis occurred among any treatment groups.

Stine et al. (2011) performed a 28 day study in which 24 barrows, eight per treatment, were fed 0.0, 1.0, or 3.3 mg/kg BW/day of melamine-cyanuric acid. Only one of eight barrows fed the 3.3 mg/kg of BW/day of melamine-cyanuric acid developed a cluster of crystals in the kidneys. No other treatment related symptoms or findings were

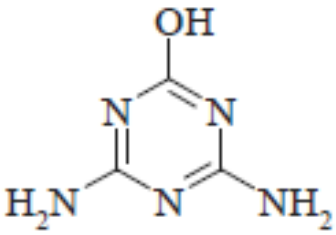
observed in any other barrows on test. For this reason a NOAEL for weanling barrows was set at 1.0 mg/kg body weight/ day of melamine plus 1.0 mg/kg body weight/day of cyanuric acid, which is equivalent to 25 ppm of each compound in the feed.

Since crystals were found in one 200 mg/kg BW/day pig in the preliminary study, Stine et al. (2011) performed a follow up study utilizing eight pigs that received 200 mg/kg BW/day melamine (approximately 5000 mg/kg of fed) while four control pigs received no melamine for 28 days. Upon examination, no crystals were present in any pigs receiving 200 mg/kg BW/day and there was no difference among treatments in any variables measured.

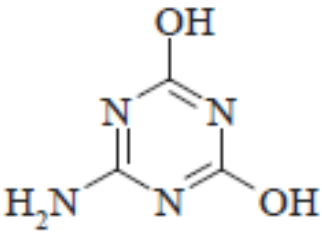
Crystal morphology from the 200 mg/kg BW/day melamine fed pig in the 7 day study by Stine et al. (2011) was closely related to crystal morphology from melamine-cyanuric acid fed pigs. Furthermore, crystal composition from melamine fed pigs and melamine-cyanuric acid fed pigs, determined by UPLC-MS/MS, was very similar. This similarity indicates that crystals from the melamine fed pigs had a melamine cyanuric acid composition of approximately one to one. Stine et al. (2011) stated that while microbial conversion of melamine to cyanuric acid, documented by Seffernick et al. (2010) and Wackett et al. (2002) does occur, it is unclear if the microorganisms responsible for the conversion reside and are active in the gastrointestinal tract of animals.



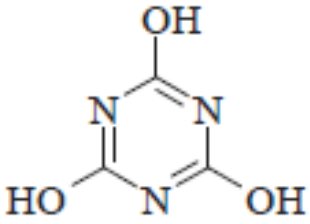
melamine



ammeline



ammelide



cyanuric acid

Figure 2.1. Structure of melamine, ammeline, ammelide, and cyanuric acid. Adapted from Tolleson (2009)

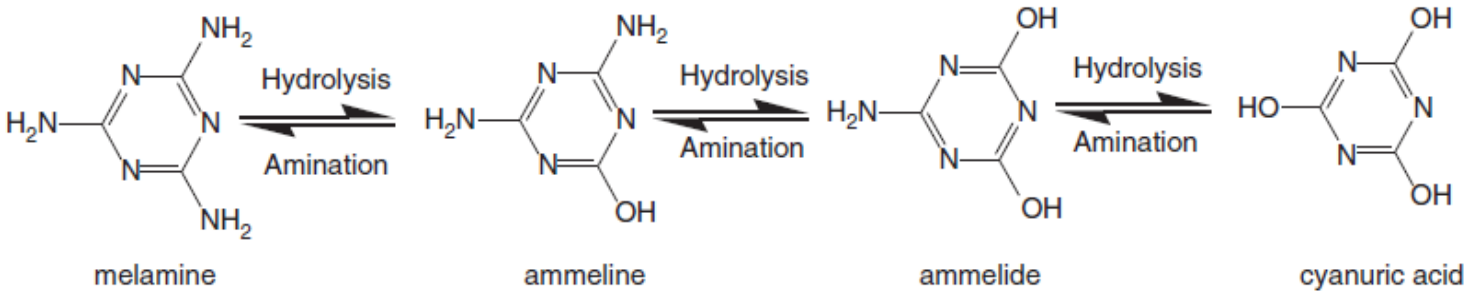


Figure 2.2. Pathway for conversion between melamine and related analogs. Adapted from Baynes and Riviere (2010).

CHAPTER 3

Effects of Melamine in Young Pekin Ducks

Introduction

Melamine ($C_3H_6N_6$) 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 melamine 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 accidently by treatment of feed ingredients with products that contain melamine (WHO, 2008a). Recently, the intentional adulteration of feeds and foods with melamine (Hilts and Pelletier, 2009) has received international attention. Melamine is 66% nitrogen (Yang et al., 2009), therefore, protein analysis using the Kjeldahl method, will result in an invalid or an over estimate of the actual protein content of a matrix that contains melamine (Yang et al., 2009). For this reason melamine was intentionally added to feed ingredients or feeds 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 was used to manufacture the pet food and it was intentionally contaminated with melamine and cyanuric acid (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. By September of the following year, some 52,875 children in China had been hospitalized after consuming infant formula contaminated with melamine (Chan et al., 2008). Some children developed renal stones that were composed of melamine and uric acid (WHO, 2008a). As a result of this incident, the World Health Organization (WHO) established a tolerable daily intake (TDI) for melamine at 0.2 mg/kg of body weight and a TDI for cyanuric acid at 1.54 mg/kg body weight (WHO, 2007).

Following the 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, 2007a), and aquaculture feed (Karbiwnyk et al., 2009). Several studies where then conducted to determine the effects of melamine and cyanuric, 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) did considerable work on the effects of melamine and cyanuric acid in young broilers and poults and reported that melamine significantly decreased the performance of broiler and poults, when included in the diet at $\geq$ 1.5%. Brand (2011) also found that the addition of cyanuric acid to a diet contaminated with melamine reduce the toxic effects of melamine in poults but not in broilers.

In an experiment by Yan et al. (2009), ducks were fed graded levels of melamine ranging from zero to 1,000 mg/kg. No melamine was detected in the breast, liver, or

kidney of ducks consuming less than 50 mg/kg of melamine in their diet for 42 days (Yan et al., 2009). However, dietary levels of melamine between 100 and 1,000 mg/kg diet resulted in tissue residues levels that increased linearly with increasing dietary levels, with ducks receiving 500 and 1,000 mg/kg having the highest concentrations of melamine in the kidneys (Yan et al., 2009).

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

The objective of the current study was to determine the effects of feeding melamine at concentrations ranging from 0 to 2.25 % of the diet to young Pekin ducks for 21 days. Additionally, the study was used to determine if melamine accumulates in the tissues of Pekin ducks, and if hepatic clearance via bile is a possible route of melamine elimination.

Materials and Methods

Diet Preparation:

A basal diet (Table 3.1.) was formulated to meet or exceed all requirements of young Pekin ducks set forth by the National Research Council (NRC, 1994). Ten dietary treatments were prepared from the basal diet by adding melamine (MEL), purchased from Fisher Scientific. MEL was substituted for sand to obtain the desired dietary MEL concentrations (0.00, 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, and 2.25 %).

Birds, Management and Response Variables:

The animal care and use protocol was reviewed and approved by the University of Missouri Animal Care and Use Committee (ACUC). Two hundred, day-old Pekin ducks were purchased from a commercial hatchery, weighed, wing banded, and assigned to pens in stainless steel batteries. A completely randomized design was used, with five replicate pens assigned to each of the ten dietary treatments. Each replicate pen contained four ducks. 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 euthanized with carbon dioxide and blood samples were collected. Following blood collection cervical dislocation was performed to ensure death by physical means. The liver and kidneys were removed from three ducks per pen and weighed. Relative liver and kidney weights were calculated by dividing organ weight by body weight. Blood samples were collected via cardiac puncture from three birds per pen, centrifuged (Sorvall, RC 3 B plus) at 1,400 x g for 30 minutes at 7ºC before serum was separated. Serum was analyzed for glucose (GLU), albumin (ALB), total protein (TP), globulin (GLOB), calcium (Ca), asparate transaminase (AST), gamma glutamyltranserase (GGT), and uric acid (UA) at the University of Missouri Veterinary Pathology Laboratory (Columbia, MO). Sections of

kidney from eight ducks per treatment were fixed in 10% neutral buffered formalin for histopathological evaluation. Sections of kidneys, breast muscle, and bile from all treatments were collected and frozen for later analysis of MEL concentrations.

Melamine Analysis:

MEL extraction from tissue and bile samples was based on the method used by Brand (2011), and involved high-performance liquid chromatography (HPLC) via UV detection. For kidney and muscle, 10 mL of water:acetonitrile (1:2) was added to 1 g of tissue and the tissues homogenized 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; Sparks, MD) and the supernatant transferred to microcentrifuge tubes and further centrifuged for 5 min at 10,000 rpm (Spectrafuge 16M; Woodbridge, NJ). The supernatant was extracted and filtered through a MycoSep® 224 AflaZon columns (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 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 d min at 10,000 rpm (Spectrafuge 16M). The supernatant was collected and filtered through MycoSep® 224 AflaZon column (Romer Labs, 2011). Finally, 500 µL of the filtered supernatant 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 fluorescence detector, Hitachi Model L-7200 autosampler with Hitachi D-7000 data acquisition interface and

ConcertChrom software on a microcomputer were used for HPLC analysis (Tokyo, Japan). A HyperClone (Phenomonex) C_{18} column (100 x 4.60 mn) was used with a retention time of 6 min and flow rate of 1 mL/min. UV detection occurred at 240 nm. The mobile phase consisted of BUFF:acetrilnitril(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 curve was calculated, plotted on the standard curve, and used to calculate individual MEL concentrations in samples. The limit of detection was set at 8.0 ppm.

Statistical Analysis:

Data were analyzed using the general linear model procedures of Statistical Analysis Software (SAS) (SAS, 2006). Pen was 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 ($y_i = a + bx_i + cx^2_i + E_i$) response. Statistical significance was accepted at a P-value of ≤ 0.05. An arcsine transformation was applied to percent mortality data before statistical analysis was performed, and a $\log_{10}$ transformation to MEL residue levels in the kidney.

Results

Performance and Mortality:

The effect of MEL on body weight gain, feed intake, feed conversion, and mortality are shown in Table 3.2. Increasing levels of MEL caused body weight gain (*P*

< 0.0001) and feed intake ($P < 0.0001$) to decrease linearly. Inclusion of MEL in the diet at ≥1.00 % caused body weight gain and feed intake to be less ($P < 0.0001$) than that of control birds. Figure 3.1 shows the size difference in Pekin ducks after consumption of MEL contaminated feed for 21 d.

Feed conversion increased in a quadratic ($P < 0.0023$) fashion with increasing dietary MEL concentrations. Feed to gain was increased ($P < 0.0001$) above that of controls in ducks that consumed diets containing ≥ to 1.50 % MEL. Percent mortality also increased in quadratic ($P < 0.0119$) fashion with increasing dietary MEL concentrations. Compared to controls, mortality was higher ($P = 0.0008$) in ducks that consumed ≥ 2.00 % MEL.

Organ Weights:

Table 3.2 summarizes the effect of MEL on relative organ weights. Increasing dietary levels of MEL caused a quadratic ($P = 0.0034$) response in relative liver weight of ducks. However, there were no differences ($P > 0.05$) in relative liver weights among controls and ducks fed ≥ 0.25 % MEL. Relative kidney weights increased linearly ($P < 0.0001$) with increasing levels of MEL, with ducks fed ≥ to 1.00 % MEL having heavier ($P < 0.0001$) relative kidney weights than control ducks. Figure 3.2 shows the difference in appearance of the kidneys from a control duck (photo 'A') and a duck fed MEL (photo 'B') for 21 days.

Serum Chemistry:

The effect of MEL on the serum chemistry of young Pekin ducks is summarized in Table 3.3. While both GLU ($P = 0.0193$) and AST ($P = 0.0021$) increased linearly as MEL levels increased, there was no difference ($P > 0.05$) among controls and ducks fed

any level of MEL. Compared to controls, ALB ($P = 0.0002$), TP ($P < 0.0001$), GLOB ($P < 0.0001$), and UA ($P < 0.0001$) were all higher in ducks fed ≥ 1.00% MEL and all increased in a linear ($P < 0.0001$) fashion with increasing dietary MEL levels. Serum GGT levels of ducks fed 1.50 and ≥ 2.00% MEL were higher ($P < 0.0001$) than levels in control ducks, and also increased in a linear ($P < 0.0001$) fashion as dietary MEL levels increased.

Tissue Residues:

Table 3.4 shows residue levels of MEL in the kidney, breast muscle, and bile as determined by HPLC. MEL residue levels in the kidney increased quadratically ($P < 0.0001$) as dietary MEL increased. Ducks fed ≥ 0.25 % MEL had MEL residue levels in the kidneys that were higher ($P < 0.0001$) than levels found in controls. Residue levels in breast muscle were higher ($P < 0.0001$) in ducks fed ≥ to 0.75% MEL than in controls. This increase in MEL levels in the breast muscle was found to be linear ($P < 0.0001$). However, at each inclusion level, MEL residue levels were lower in the breast muscle than in the kidney. Due to logistical problems during termination, bile from ducks in each treatment were accidentally pooled. Therefore, statistical analysis could not be performed on the bile data. Means presented in Table 3.4 are averages of duplicate HPLC analysis performed on bile samples taken from each treatment. Residue levels in the bile increased as dietary inclusion increased, with a low of 25 ppm in ducks fed 0.25 % MEL and a high of 640 ppm in ducks fed 2.25 %.

Pathology:

Gross Pathology –Mortality:

Table 3.2 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 hemorrhaging and trauma and appeared to be unrelated to the treatment. 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. One mortality occurred in the 1.75 % MEL treatment group and presented with pale enlarged kidneys. The 2.00 and 2.25 % MEL treatment groups had four mortalities each. All but one duck from 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 3.3 shows crystals present in the bile from a duck fed control diet with MEL (photo 'B'), while photo 'A' shows the clear bile from a duck fed the control diet.

Histopathology – Mortality:

Histopathologic examination of the kidneys from early mortalities occurring in the 1.50 % and higher MEL groups revealed moderate to severe autolysis in all sections with numerous basophilic mineralized casts noted within the renal tubules and collecting ducts. The renal lesions noted in these mortalities were compatible with MEL toxicity seen in other avian species.

Gross Pathology – Termination:

By the end of the 21 day study there was clear size difference between ducks from the control group and ducks fed diets that were contaminated with high levels of MEL (Figure 3.1). Gross examination of ducks that received diets with high levels of MEL (≥ 1.0 % MEL) revealed enlarged and pale kidneys (Figure 3.2).

Histopathology – Termination:

Microscopic examination of the pooled bile specimens collected during termination revealed a moderate number of one to ten micrometer spherical brown crystals with numerous aggregated crystals in treatments fed 1.00 % MEL and ≥ 1.50 % 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 3.4 shows crystals present in the bile of ducks fed 2.25 % MEL for 21 days.

Histopathology of kidney sections from treatments fed 0.00, 0.25, and 0.50 % MEL were unremarkable. Two kidneys from the 0.75 % treatment group had mild dilation of the embryonal nephrons with eosinophilic to basophilic casts present. Several kidney sections from each treatment fed ≥ 1.00 % MEL contained eoinophilic 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 heterphil infiltration present in kidney sections of ducks fed ≥ 1.00 % MEL (Figure 3.5). 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 golmeruli. These finding suggest mild to severe melamine toxicity in treatments fed ≥ 2.00 % MEL.

Discussion

Compared to controls, mortality was higher in ducks fed ≥ 2.00 % MEL. Brand (2011) reported poults fed ≥ 1.5 % MEL and broilers fed ≥ 2.5 % MEL to have significantly higher mortality than controls. In the current study, body weight gain and feed intake were both reduced when ducks consumed diets that contained ≥ 1.00 % MEL. These results are consistent with research by Brand (2011), in which broilers and poults that consumed ≥ 1.00 % MEL had decreased body weight gains. 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 documented in poults fed up to 1.50% MEL and survived for the 21 day experimental period. When feeding up to 100 mg/kg (0.01 % of the diet) MEL to laying ducks for 42 days, Gao et al. (2010) did not observe any negative effect on body weight gain, feed intake or feed conversion. Data from the current study shows that ≤ 0.75 % MEL in the diet does not reduce performance of young Pekin ducks. Data also shows that MEL does not cause significant mortality until inclusion is ≥ 2.00 % of the diet. However, it should be noted that ducks fed 1.50% MEL had 15% mortality (not different ($P > 0.05$) from controls) with histopathological examination of the kidneys revealing renal lesions compatible with MEL toxicity leading to mortality.

Relative liver weights responded in a quadratic fashion as dietary melamine levels increased. Brand (2011) report heavier relative liver weights in young broilers fed ≥ 2.25 % MEL, which is higher than levels used in the current study. A linear increase in relative liver weight was also documented in poults fed up to 1.5 % MEL and survived to termination (Brand, 2011). Relative kidney weight increased linearly as dietary levels of MEL increased. The kidneys were found to be heavier than the controls in ducks fed ≥ 1.00 % MEL. Increased relative kidney weights have been observed in studies with broilers fed ≥ 1.50 % MEL and poults fed ≥ 1.00 % MEL (Brand, 2011). Gao et al. (2010) fed 100 mg/kg of MEL (0.01 % of the diet) to laying Jinding ducks and observed heavier relative kidney weights after 21 days on test. These data suggest a great deal of variability in how different types and ages of birds 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 needed to significantly affect body weight gain, feed intake, and relative kidney size.

All blood parameters 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 increase. Brand (2011) documented 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 was documented in poults fed up to 1.50 % MEL (Brand, 2011). However, poults receiving 2.00, 2.50, and 3.00 % MEL were terminated early due to high treatment related mortality, and blood serum was not collected for analysis.

Elevated UA can be used to diagnose renal failure, with increased levels occurring when more than 70 % of kidney function is lost (Cornell, 2010c). In the present study, ducks consuming 2.25 % MEL had UA levels that were 4.7 times that of control ducks. GGT is an enzyme present in the liver, kidney and pancreas (Nicoll et al., 2012) that catalyzes the transfer of amino acids from one peptide to another (Vroon and Israili, 1990). Elevated levels of GGT can be also an indication of renal failure (Limdi and Hyde, 2003). Therefore, elevated levels of UA and GGT in the current study point towards a decrease in renal function.

Dehydration could explain the elevated levels of ALB, TP, GLOB, and GLU. Dehydration can occur due to the loss of appetite and nausea associated with kidney failure (early mortality ducks fed ≥ 2.00 % MEL had little to no feed in the upper gastrointestinal tract). Another possible cause of dehydration is the diuretic effect of MEL, which has been documented in dogs (Lipschitz and Stockey, 1945). Serum concentrations of ALB, TP, and GLOB can all be elevated during periods of dehydration (Cornell, 2010a; Nicoll et al., 2012). This concentration of solids in the blood, which is usually the result of fluid loss to the tissues, is termed hemoconcentration (Merriam-Webster, 2012). The occurrence of hemoconcentration has been reported during periods of dehydration (Diseases, 2008). The measurement of packed cell volume could be used to confirm these results, but was not measured in the current experiment.

Asparate transaminase (AST), an enzyme that catalyzes the transfer of alpha amino groups from aspartic acid to alpha-ketoglutaric acid (Cornell, 2010b) was found to increase in a linear fashion during the current experiment. AST is not organ specific but is known to be found in renal epithelial cells (Cornell, 2010b). Therefore, damage to the

epithelial cell could result in release of AST into the blood, thus elevating serum AST levels in ducks fed higher dietary levels of MEL.

MEL residue in the kidney increased in a quadratic fashion. Ducks fed ≥ 0.25 % MEL had residue levels in the kidney that were higher than controls. Ducks fed 2.25 % MEL had kidney residue concentration of 295 mg/kg. Broilers fed 2.25 % MEL for 21 days had kidney residue values of 846 ppm (Brand, 2011) which is 2.8 fold higher than residue levels found in the kidneys of ducks fed 2.25 % MEL for 21 days. Brand (2011) fed 2.25 % MEL to poults and reported kidney MEL concentrations of 586 mg/kg. This twofold difference between poults and ducks could be due to the differences in how the two species absorb and eliminate MEL. Extremely high mortality (≥ 63 %) was observed by Brand (2011) when feeding ≥ 2.00 % MEL in the diet to poults. In the current study, mortality was only 20 % for ducks fed 2.00 and 2.25 % MEL. These data suggest that ducks, unlike poults are able to excrete MEL more efficiently. Thus MEL does not accumulate in the kidney of ducks to the degree it does in poults and renal related mortality is reduced [32 % in poults (Brand, 2011) and 15 % in ducks: both fed 1.50 % MEL].

Muscle residue levels were lower than residue levels seen in the kidney. This is similar to data reported by Lu et al. (2009), in which the kidneys of broilers fed 0.1 % MEL for 42 days had a residue value of 9.17 ppm, which is higher than the 3.73 ppm detected in the breast meat of ducks from the same treatment. Higher MEL residue values in the kidney than the breast muscle is supported by data by Baynes et al. (2008) who suggested MEL distribution in the pig was related to total body water and is not distributed to most organs, and Dobson et al. (2008) and Puschner et al. (2007) who noted

precipitation of MEL and CYA complexes in the kidney. Precipitation of MEL and CYA 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. In the current study, bile had a much higher concentrations of MEL than the kidney, suggesting that bile is a route of elimination in Pekin ducks. Use of the bile as a route of elimination has been suggested to occur in other avian species by Brand (2011).

Gross and histopathology of early mortalities revealed that birds fed ≥ 1.50 % MEL treatments to have been off feed at time of death. All but one of these ducks had pale and enlarged kidneys. Enlarged and pale kidneys have been documented in poults and broilers fed ≥ 1.00 and ≥ 2.00 % MEL, respectively (Brand, 2011). The renal lesions noted in early mortality ducks were comparable to those seen with MEL toxicity in broilers and poults (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 affect the kidney until dietary inclusion is ≥ 0.75 %. Dietary levels between 0.75 % MEL to 1.75 % MEL caused renal damaged compatible with mild toxicity. Dietary MEL ≥ 2.00 % caused renal damage compatible with mild to severe toxicity. The pathology documented in ducks fed ≥ 0.75 % MEL is comparable to that documented in chickens and turkeys (Brand, 2011). Mature Jinding ducks exposed to 25 mg/kg of fed (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).

It appears that concentrations of MEL in the bile need to be > 203 ppm to favor crystal formation. This is based on the residue levels of MEL detected in the bile via HPLC analysis. The bile of ducks fed 0.75 % MEL had a residue value of 203 ppm and no crystals visible during microscopic examination while ducks fed 1.00 % MEL had bile residue values of 475 ppm and a moderate number of crystals present in the bile. Histopathology evaluation revealed that 1.00 % MEL was the lowest dietary treatment to induce crystals in the kidney. At this level, HPLC analysis determined a MEL residue level of 335 ppm in the kidney. The next lowest treatment, 0.75 % MEL, had a kidney residue value of 161 ppm. This suggests that melamine concentrations need to exceed 161 ppm to induce crystal formation in the kidney.

In 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 renal failure induced by MEL damage is the most likely cause of 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 ≥ 1.00 % MEL, along with changes in serum ALB, TP, GLOB, and UA, at the same level, it appears that dietary concentrations ≤ 0.75 % is the threshold level that can be tolerated, without significant negative effects, on young Penkin ducks. Finally, HPLC analysis shows that the bile is used as a way for ducks to eliminate MEL from their body.

Table 3.1. Ingredients and nutrient composition of basal ration used to make individual experimental diets for young Pekin ducks

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
None Phytate Phosphorus (% Av.)	0.40

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

Table 3.2. Effects of melamine on performance and organ weights of Pekin ducks

Treatment[1]		Response Variables[2]					
Melamine (%)		BWG (g)	FI (g)	F:G (g:g)	Mortality[3] (%)	Liver[4] (%)	Kidney[4] (%)
Basal Diet + 0.00% M		832[a]	1,658[a]	2.01[d]	5[bc]	3.89	1.18[d]
Basal Diet + 0.25% M		787[a]	1,647[a]	2.09[d]	0[c]	3.84	1.20[d]
Basal Diet + 0.50% M		774[a]	1,590[a]	2.05[d]	0[c]	4.04	1.32[cd]
Basal Diet + 0.75% M		775[a]	1,557[a]	2.04[d]	5[bc]	4.08	1.26[cd]
Basal Diet + 1.00% M		615[b]	1,324[b]	2.17[d]	0[c]	4.23	1.95[bc]
Basal Diet + 1.25% M		615[b]	1,361[b]	2.22[d]	0[c]	4.12	1.94[c]
Basal Diet + 1.50% M		458[c]	1,128[c]	2.65[c]	15[ab]	4.00	2.64[ab]
Basal Diet + 1.75% M		439[cd]	1,146[c]	2.73[bc]	5[bc]	3.97	2.84[a]
Basal Diet + 2.00% M		358[d]	1,028[c]	3.12[a]	20[a]	3.67	3.30[a]
Basal Diet + 2.25% M		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 = feed intake; F:G = feed to gain; M = melamine.

Table 3.3. Effects of melamine on serum chemistry 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% M		224	1.32^{c}	3.12^{c}	1.82^{c}	53.4	2.68^{de}	7.34^{d}
Basal Diet + 0.25% M		226	1.32^{c}	3.10^{c}	1.78^{c}	36.9	2.32^{e}	8.62^{d}
Basal Diet + 0.50% M		184	1.34^{bc}	3.26^{c}	1.90^{c}	38.5	2.48^{e}	7.78^{d}
Basal Diet + 0.75% M		199	1.34^{bc}	3.30^{c}	1.98^{c}	28.0	2.54^{e}	11.56^{cd}
Basal Diet + 1.00% M		263	1.62^{a}	3.96^{ab}	2.40^{ab}	29.5	3.86^{bcd}	25.10^{ab}
Basal Diet + 1.25% M		239	1.50^{ab}	3.80^{b}	2.30^{b}	73.4	3.00^{cde}	21.44^{bc}
Basal Diet + 1.50% M		252	1.50^{ab}	3.98^{ab}	2.46^{ab}	99.7	4.02^{bc}	31.62^{ab}
Basal Diet + 1.75% M		248	1.54^{a}	4.10^{ab}	2.54^{a}	57.3	3.86^{bcd}	35.48^{a}
Basal Diet + 2.00% M		255	1.58^{a}	4.10^{ab}	2.52^{a}	239.8	4.74^{ab}	33.84^{a}
Basal Diet + 2.25% M		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; M = melamine.

Table 3.4. Residue levels of melamine in kidney, muscle, and bile of Pekin ducks

Treatment[1]		Response Variables		
Melamine (%)		Kidney[2] (ppm)	Muscle[3] (ppm)	Bile[4] (ppm)
Basal Diet + 0.00% M		ND[e]	ND[f]	0
Basal Diet + 0.25% M		29[d]	14[f]	25
Basal Diet + 0.50% M		42[c]	13[f]	91
Basal Diet + 0.75% M		161[b]	74[e]	203
Basal Diet + 1.00% M		335[a]	127[d]	475
Basal Diet + 1.25% M		284[a]	135[cd]	427
Basal Diet + 1.50% M		385[a]	212[b]	613
Basal Diet + 1.75% M		388[a]	184[bc]	575
Basal Diet + 2.00% M		340[a]	173[bcd]	640
Basal Diet + 2.25% M		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 ($P < 0.05$).
M = melamine; ND = none detected.

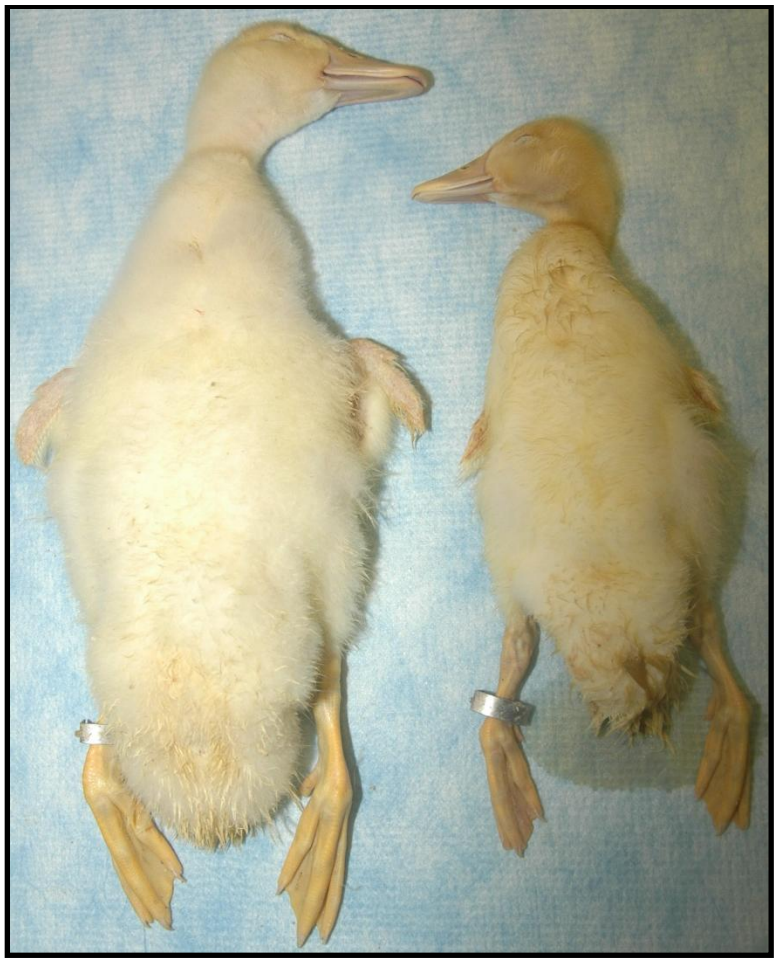


Figure 3.1. Effects of melamine on the performance of young Pekin ducks fed treatments from hatch to 21 days of age. Duck on left belong to the control treatment while the duck on the right received a diet contaminated with MEL.

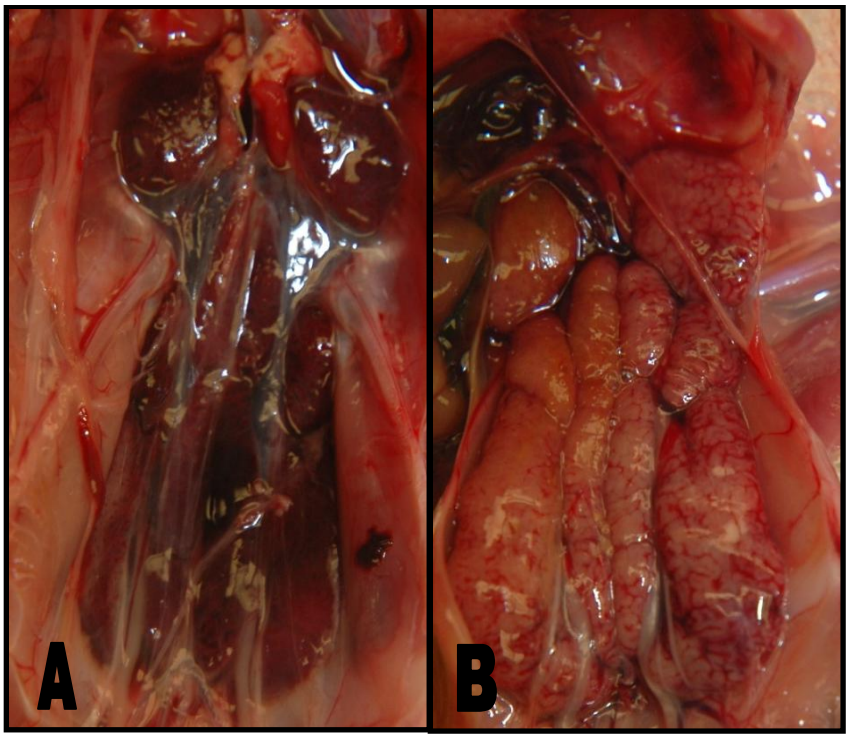


Figure 3.2. Effect of melamine on the kidneys of young Pekin ducks fed treatments from hatch to 21 days of age. A) Kidney of control duck. B) Enlarged and pale kidney of duck fed MEL.

Figure 3.3. Effect of melamine on the bile of young Pekin ducks fed treatments from hatch to 21 days of age. A) Bile from a duck fed control diet. B) Bile from a duck fed MEL. Cloudy appearance of bile in photo 'B' is due to presence of white crystal precipitate.

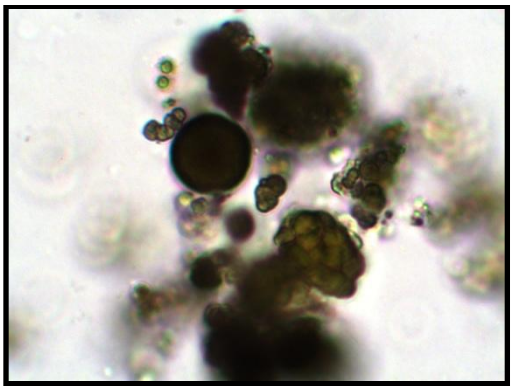


Figure 3.4. Microscopic examination of bile from ducks fed graded levels of melamine. Crystals observed at 400 x magnification in the bile of duck fed 2.25 % MEL for 21 days.

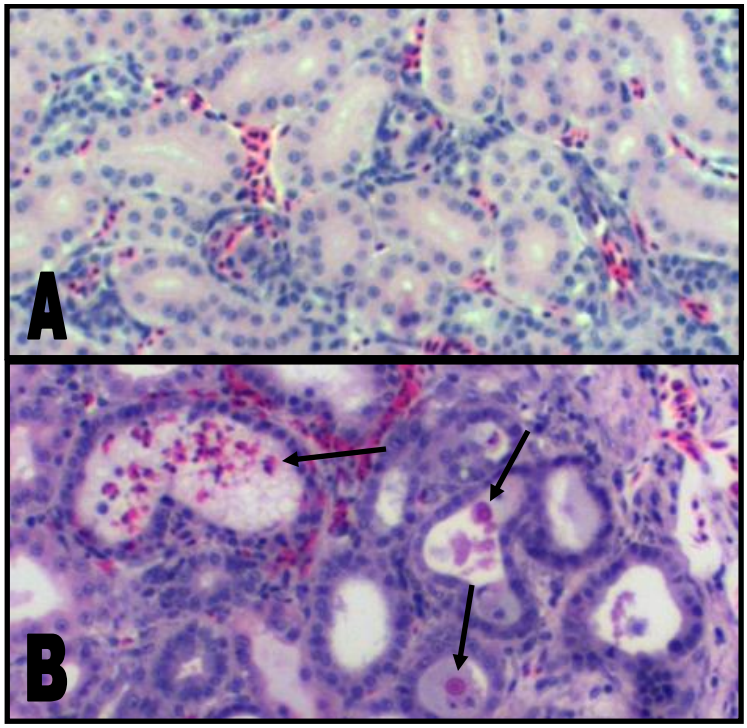


Figure 3.5. Kidney section from a control and duck fed 2.25 % melamine, viewed at 100 x magnification. Photo 'A' shows the appearance of a normal kidney section while photo 'B' shows the effects of 2.25 % MEL on the kidney of a young Pekin duck from hatch to 21 days of age. Arrows point to dilated tubules with casts present.

Chapter 4

Individual and Combined Effects of Melamine and Cyanuric Acid in Young Pekin Ducks

Introduction

Melamine ($C_3H_6N_6$) 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, textiles finishes, and fertilizers (Hilts and Pelletier, 2009). Melamine is 66% nitrogen by mass and will artificially increase the protein content of a matrix when the Kjeldahl method is used for protein analysis (Yang et al., 2009). Therefore, melamine has been added to feed ingredients to increase their apparent crude protein value and subsequently their monetary value (WHO, 2008a). Cyanuric acid, along with ammeline and ammelide, are structural analogues of melamine (Tyan et al., 2009; WHO, 2008a) and all belong to the class of chemicals known as *s-triazines* (Wackett et al., 2002). Hydrolysis or amination of one *s-triazine* can result in the production of another *s-triazine* (Baynes and Riviere, 2010).

Conversion of melamine to cyanuric acid can occur during the manufacturing of melamine resulting in a final product that is not 100% pure (Dobson et al., 2008). Jutzi et al. (1982) documented that bacteria can convert melamine to cyanuric acid with Filigenzi et al. (2007) stating that no known conversion of melamine to related compounds exist in mammals. However, Baynes and Riviere (2010) state that bacterial degradation could

possibly take place in the gastrointestinal tract or biological fluids. Therefore, there exist several possible routes by which both melamine and related compounds can be found in biological systems simultaneously.

In 2007, the deaths of cats and dogs related to renal failure were documented across North America and South Africa (Hilts and Pelletier, 2009). It was later determined that wheat gluten, imported from China and incorporated into pet food, had been contaminated with melamine and cyanuric acid (Hilts and Pelletier, 2009). Brown et al. (2007) examined six dogs and ten cats that consumed contaminated pet food and observed that all had elevated blood urea nitrogen and creatinine levels with polarizable crystals in the distal tubules and collecting ducts of their kidneys.

Toxicity in mammals occurs because of the ability of melamine and cyanuric acid to self-assemble and form a hydrogen-bonded bimolecular network (Perdigao et al., 2006). Self-assembly can lead to the formation of insoluble compounds that can precipitate in the kidneys, leading to renal failure (Seffernick et al., 2010). Publications by Tolleson (2009) and Dobson et al. (2008) described possible routes of absorption of melamine and related compounds as well as precipitation of insoluble compounds which can lead to renal failure.

Following the incident of contamination of pet food in 2007, it was determined that waste material containing melamine and cyanuric acid from pet food manufacturing was incorporated into swine, poultry, and aquaculture feeds (Buur et al., 2008; Karbiwnyk et al., 2009; USDA, 2007a). The Food Safety and Inspection Service (FSIS) division of the USDA, in cooperation with state and local producers, quarantined all animals that had consumed contaminated pet food scraps (USDA, 2007a). However,

after extensive testing, the USDA FSIS determined that products from poultry and swine which consumed feed contaminated with melamine and related compounds were "not adulterated" and were able to be offered for sale (USDA, 2007a, b). Since the contamination of pet food and subsequent contamination of swine and poultry feed, several publications have reported on the effects of consumption of both melamine and cyanuric acid, alone or in combination, to poultry and swine (Brand, 2011; Reimschuessel et al., 2008; Stine et al., 2011).

Brand (2011) conducted six experiments to determine the effects of feeding melamine and cyanuric acid, alone or in combination, to broilers and poults from hatch to 21 days of age. It was determined that melamine alone depressed growth performance of young broilers and poults when it was included in the diet at ≥ 1.5% (Brand, 2011). Inclusion of up to 3.0 % cyanuric acid to young broiler and poult diets did not affect performance or kidney function (Brand, 2011). The inclusion of cyanuric acid to diets that contained melamine (0.5, 1.0, and 1.5% of each compound) reduced the negative effects of melamine in poults (Brand, 2011). However, in young broilers, the combination of cyanuric acid and melamine resulted in poorer growth performance and production of crystals in the kidneys as compared to either compound alone (Brand, 2011).

The objective of the current study was to determine the effects of feeding melamine and cyanuric acid, alone or in combination, to Pekin ducks from hatch to 21 days of age. Dietary treatments included melamine and cyanuric acid, individually at 0.5, 1.0, and 1.5% of the diet, and combinations of 0.5, 1.0, and 1.5% of both compounds. Another objective of this study was to determine if melamine accumulated in the tissues

of Pekin ducks, and if hepatic clearance via bile is a possible routes of melamine elimination.

Materials and Methods

Diet Preparation:

A basal diet (Table 4.1) was formulated to meet or exceed all requirements set forth by the Nation Research Council for young Pekin ducks (NRC, 1994). Ten dietary treatments were prepared by adding melamine (MEL; Fisher Scientific) or cyanuric acid (CYA; Fisher Scientific) to the basal diet. MEL and CYA were substituted for sand to obtain the desired dietary concentrations: 1) Control, no MEL or CYA (basal diet); 2) basal diet + 0.50% MEL; 3) basal diet + 1.00% MEL; 4) basal diet + 1.50% MEL; 5) basal diet + 0.50% CYA; 6) basal diet + 1.00% CYA; 7) basal diet + 1.50% CYA; 8) basal diet + 0.50% MEL and 0.50% CYA; 9) basal diet + 1.00% MEL and 1.00% CYA; and 10) basal diet + 1.50% MEL and 1.50% CYA.

Birds, Management, and Response Variables:

The animal care and use protocol was reviewed and approved by the University of Missouri Animal Care and Use committee (ACUC). Two hundred and twenty three day-old Pekin ducks were purchased from a commercial hatchery, weighed, wing banded, and assigned to pens in stainless steel batteries. A completely randomized design was used. Controls and treatments fed MEL alone or MEL + CYA consisted of five replicates with five ducks per replicate group. Treatments fed CYA alone consisted of four replicates with four ducks per replicate group. 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 days. 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 body weight gain, feed intake, and feed conversion were calculated. All ducks were then euthanized by carbon dioxide followed by cervical dislocation. Blood samples were collected, via cardiac puncture, from ten ducks per treatment, centrifuged (Sorvall, RC 3 B plus) at 1,400 x g for 30 minutes at 7ºC before serum was separated. Serum was then sent to the University of Missouri Veterinary Pathology Laboratory (Columbia, MO) for analysis of glucose (GLU), albumin (ALB), total protein (TP), globulin (GLOB), calcium (Ca), asparate transaminase (AST), gamma glutamyltranserase (GGT), and uric acid (UA). Liver and kidneys were removed from three ducks per pen and weighed. Relative liver and kidney weights were calculated by dividing organ weight by body weight. Sections of liver and kidney were taken from six ducks per treatment and fixed in 10% neutral buffered formalin for histopathological evaluation. Sections of liver, kidney, and breast muscle, along with bile from all treatments, were collected and frozen for later analysis of MEL concentrations.

Melamine Analysis:

MEL extraction from tissue and bile samples was based on the method used by Brand (2011), and involved high-performance liquid chromatography (HPLC) via UV detection. For kidney and muscle, 10 mL of water:acetonitrile (1:2) was added to 1 g of tissue and the tissues homogenized 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;

Sparks, MD) and the supernatant transferred to microcentrifuge tubes and further centrifuged for 5 min at 10,000 rpm (Spectrafuge 16M; Woodbridge, NJ). The supernatant was extracted and filtered through a MycoSep® 224 AflaZon columns (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 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 d min at 10,000 rpm (Spectrafuge 16M). The supernatant was collected and filtered through MycoSep® 224 AflaZon column (Romer Labs, 2011). Finally, 500 µL of the filtered supernatant 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 fluorescence detector, Hitachi Model L-7200 autosampler with Hitachi D-7000 data acquisition interface and ConcertChrom software on a microcomputer were used for HPLC analysis (Tokyo, Japan). A HyperClone (Phenomonex) C_{18} column (100 x 4.60 mn) was used with a retention time of 6 min and flow rate of 1 mL/min. UV detection occurred at 240 nm. The mobile phase consisted of BUFF:acetrilnitril(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 curve was calculated, plotted on the standard curve, and used to calculate individual MEL concentrations in samples. The limit of detection was set at 8.0 ppm.

Statistical Analysis:

Data were analyzed using the general linear model procedures of Statistical Analysis Software (SAS, 2006). Pen was the experimental unit. Variables that showed significant differences in the ANOVA were compared using Fisher's protected least significant difference procedure. Statistical significance was accepted at a *P*-value of $\leq$ 0.05. An arcsine transformation was applied to percent mortality data before statistical analysis was performed. Data for MEL residue levels in the muscle tissue and kidneys were ranked, based on a method developed by Conover and Iman (1981), before analysis.

Results

Performance and Mortality:

The effects of MEL and or CYA on body weight gain, feed intake, feed conversion, and mortality are summarized in Table 4.2. Inclusion of MEL at 0.50 % of the diet did not negatively ($P > 0.05$) affect body weight gain, average feed intake, or percent mortality. However, ducks fed MEL at 1.00 and 1.50 % of their diet had lower ($P < 0.0001$) body weight gains and feed intake, and higher percent mortality than control ducks. Inclusion of 1.50 % MEL in the diet increased ($P < 0.0002$) feed to gain compared to that of control birds. Figure 4.1 demonstrates the effect of 1.50 % MEL (duck 'D') on body gain weight of ducks up to 21 days of age.

The addition of CYA up to 1.50 % of the diet did not cause body weight gain, average feed intake, feed conversion, or mortality to be different ($P > 0.05$) from that of control ducks. The inclusion of CYA to diets that contained MEL, in a 1:1 ratio of CYA to MEL, was able to reduce the negative effects of MEL on performance. There was no

difference ($P > 0.05$) among controls and birds fed combinations of MEL and CYA, up to 1.50 % of each compound, in body weight gain, average feed intake, feed conversion, or percent mortality. Figure 4.1 shows the ability of CYA, added to a diet that contains MEL (duck 'J'), to alleviate the effects on body weight gain MEL (duck 'D').

Organ Weights:

The effects of MEL, CYA and combinations of MEL + CYA on organ weights of young Pekin ducks are summarized in Table 4.2. There was no effect ($P > 0.05$) of MEL or CYA, alone or in combination, on relative liver weights of ducks. Ducks fed 1.0 and 1.50 % MEL had heavier ($P < 0.0001$) relative kidney weights than controls. Ducks fed CYA up to 1.5 % of the diet, and CYA in combination with MEL, did not have relative kidney weights that were different ($P > 0.05$) from that of controls. Figure 4.2 shows the effect of MEL (1.50 %; picture 'B') and MEL + CYA (1.50 % MEL + 1.50 % CYA; picture 'C') on the kidneys of young Pekin ducks. Picture 'A' shows the kidneys of ducks fed a control diet, which are similar to the kidneys of ducks fed MEL + CYA (picture 'C'). However, the kidneys from ducks fed MEL (1.50 %; picture 'B') are pale in enlarged when compared to the kidneys from control ducks (picture 'A') and ducks fed MEL + CYA (picture 'C').

Serum Chemistry:

Table 4.3 shows the effects of MEL and CYA, alone or in combination, on the serum chemistry of young Pekin ducks. Serum concentrations of GLU, Ca, AST, and GGT were not affected ($P > 0.05$) by any treatment. Serum ALB ($P = 0.0004$), TP ($P = < 0.0001$), GLOB ($P = < 0.0001$), and UA ($P < 0.0001$) were elevated above control levels

in ducks fed 1.00 and 1.50% MEL in the diet. No other treatments caused a change ($P > 0.05$) in levels of these serum parameters.

Tissue Residue:

The effects of MEL and CYA alone or in combination on the residue levels of MEL in the kidneys, breast muscle, and bile of the ducks can be seen in Table 4.4. Inclusion of MEL at ≥ 0.50 % of the diet increased concentrations of MEL in kidneys ($P < 0.0001$) and breast muscle ($P < 0.0001$) compared to control ducks. No MEL was detected in the kidneys or breast muscle of ducks fed ≤ 1.50 % CYA. Combination (MEL + CYA) fed ducks had lower MEL residue levels in their kidneys and breast muscle compared to ducks fed MEL (≤ 1.50 %) alone. Ducks fed the highest combination of MEL + CYA (1.50 % of each compound) had no MEL residue in their kidneys or breast muscle. Ducks fed lower concentrations of MEL + CYA (≤ 1.00 % of each compound) had residue levels that were higher than ducks fed 1.50 % MEL + 1.50 % CYA, but approximately four fold lower than ducks fed 0.50% MEL alone.

Due to logistical problems during termination, bile from birds in each treatment were pooled. Therefore, statistical analysis could not be performed on the bile. Means presented in Table 4.4 are an average of duplicate HPLC analysis performed on bile samples taken from each treatment. MEL levels in the bile increased as more MEL was included in the diet, ranging from 92 ppm in ducks fed 0.50 % MEL to 568 ppm in ducks fed 1.50 % MEL. Combination fed ducks (MEL + CYA) had MEL levels in the bile ranging from 23 to 30 ppm. While these levels are higher than controls, they are much lower than in ducks fed MEL only.

Pathology:

Mortality:

The percent mortality can be seen in Table 4.2. Only diets that included MEL alone (0.50, 1.00, 1.50 %) incurred mortality at one, four, and eleven percent, respectively. Eleven of the thirteen ducks that were examined had pale and enlarged kidneys. Eight of the thirteen ducks had white crystals present in the bile during gross examination. Figure 4.3 shows crystals present in the bile of a duck fed 1.50 % MEL. Crystals from the bile of early these were spherical and brown in appearance.

Termination:

By day 21 of the study, there was a clear difference in size of ducks among treatments. Ducks that consumed 1.00 and especially 1.50 % MEL were much smaller than control ducks and ducks fed ≤ 1.50 % CYA (Figure 4.1). Microscopic examination of pooled bile specimens collected during termination revealed isolated spherical crystals present in the bile of ducks fed 1.00 % MEL, and numerous brown small spherical crystals, with some aggregates of spherical crystals present in the bile of ducks fed 1.50 % MEL. Bile from all other groups appeared clear and without crystals when viewed microscopically. Figures 4.4 and 4.5 show crystals in the bile of ducks fed 1.00 and 1.50 % MEL.

Histopathology of liver sections from all treatment groups were unremarkable. Ducks fed 1.00 and 1.50 % MEL were the only treatments to have kidneys that showed abnormalities during histopathological examination. Four kidneys sections from the 1.00 % MEL treatment group and five kidneys from the 1.50 % MEL treatment group had mild dilation of the embryonal nephrons and collecting tubules. Eosinophilic to

basophilic casts, some containing spherical eosinophilic crystals were also present in the embryonal nephrons and collecting tubules (Figure 4.6). One kidney from the 1.50 % MEL treatment had moderate dilation of the embryonal nephrons and collecting tubules with eosinophilic to basophilic casts, some with spherical eosinophilic crystals, present. Affected nephrons were often surrounded by a mild heterophilic infiltration. In rare occasions, casts or crystals, similar to the ones previously described, were found in the interstitial spaces and associated with mild multifocal hetrophil infiltration (Figure 4.7).

Discussion

Only treatments fed MEL (0.50, 1.00, and 1.50 %) incurred mortality during the current experiment. In a previous study, 15 % mortality was observed in ducks fed 1.50 % MEL (chapter 3). This mortality rate is lower than the 44 % mortality that occurred in ducks fed 1.50 % MEL in the current study. Fifty six percent mortality was reported in young poults receiving 1.50 % MEL for 21 days (Brand, 2011). However, Brand (2011) reported no treatment related mortality when feeding up to 1.50 % MEL to young broilers for 21 days. Lu et al. (2009) documented no visible signs of ill health, mortality rates, or changes in the behavior of broilers fed graded levels of MEL up to 0.1 % of the diet for 42 days. Mortalities from the current study were MEL related with ducks having pale and enlarged kidneys. Pale and enlarged kidneys as a result of MEL ingestion has been reported in poults fed ≥1.00 MEL, early mortality broilers fed ≥ 2.00 % MEL (Brand, 2011), and in ducks fed ≥ 1.50 % MEL (Chapter 3).

In the current study, no mortality occurred in the control treatment or in ducks fed CYA alone or MEL + CYA. Similar results were reported when feeding up to 3.00 %

CYA to young broiler and poults (Brand, 2011). Brand (2011) also reported no significant difference in mortality rates between controls and young broilers fed a combination of MEL and CYA (1.50 % of each compound).

Decreases in performance (body weight gain, feed intake, feed conversion) are in agreement with results from chapter 3 where body weight gain and feed intake were decreased in ducks fed ≥ 1.00 % MEL and feed conversion increased in ducks fed ≥ 1.50 % MEL. The reduction in body weight gain is consistent with research in broilers and poults that consumed ≥ 1.00 % MEL (Brand, 2011). Feed intake was also reduced in young broilers fed ≥ 1.00 % MEL for 21 days (Brand, 2011). Results of the current study support results of Lu et al. (2009), who noted no effects on weight gain, feed intake, and feed conversions of broilers fed up to 0.1 % MEL for 42 consecutive days.

Inclusion of up to 1.50 % CYA did not cause weight gain, feed intake, or feed conversion to be different than that of control ducks. Inclusion of up to 3.00 % CYA in the diets of young broilers and poults also did not cause weight gain, feed intake, or feed conversion to be different from that of controls (Brand, 2011). Similar results were observed in ducks fed combinations of MEL + CYA (up to 1.50 % of each compound), with weight gain, feed intake and feed conversion being similar to controls. These results are similar to those reported in poults, where the inclusion of CYA to a diet that contained MEL (up to1.50 % of each compound) reduced the negative effects of MEL with respect to body weight gain and feed intake (Brand, 2011) . In contrast to the findings in the current study and the findings in poults by Brand (2011), broilers that consumed a combination of MEL and CYA (up to 1.50 % of each compound) had poorer

performance (feed intake and body weight gain) than broilers fed MEL (up to 1.50 %) or CYA (up to 1.50 %) alone (Brand, 2011).

Relative liver weights were unaffected in the current study. However, relative kidney weights were increased in ducks fed ≥ 1.00 % MEL. Similar results are reported in Chapter 3, where feeding ≥ 1.00 % MEL to ducks increased kidney weights above that of controls. Increased relative kidney weight have also been observed in studies with broilers fed ≥ 1.50 % MEL and poults fed ≥ 1.00 % MEL (Brand, 2011). Feeding CYA alone or MEL + CYA did not cause relative kidney weights to differ from controls. Brand (2011) reported similar finding in poults, where feeding a combination of MEL and CYA (up to 1.50 % of each compound) did not increase their relative kidney weights. However, the ability of CYA to reduce the negative effects of MEL on relative kidney weights, observed in the current study and in poults (Brand, 2011), was not observed when feeding combinations of MEL and CYA (up to 1.50 % of each) to young broilers (Brand, 2011).

From the data discussed previously (mortality, performance, and organ weight) ≤ 1.50 % CYA in a duck ration does not affect mortality, performance, or relative liver and kidneys weight. The addition of ≥ 1.00 % MEL does cause a reduction in feed intake, body weight gain, percent mortality, and increased relative kidney weight. However, when ≤ 1.50 % CYA is added to a diet that contains MEL (one-to-one ratio of CYA to MEL), CYA is able to reduce the negative effects of MEL on feed intake, body weight gain, percent mortality, and relative kidney weights. This ability of CYA to reduce the negative effects of MEL is in agreement with research in poults fed similar dietary concentrations (Brand, 2011). However, these data are in contrast to findings in broilers

fed similar dietary concentrations where the combination of MEL and CYA was more toxic that MEL alone. Data from the current experiment are also in contrast to swine with respect to gain (Stine et al., 2011) where the combination of MEL and CYA (100 mg/kg BW/day, of each compound) caused a greater reduction in gain than MEL (200 mg/kg BW/day) alone.

Dehydration could explain the elevated levels of ALB, TP, and GLOB that were observed in ducks fed ≥ 1.00 % MEL during the current study. Dehydration can occur due to the loss of appetite and nausea associated with kidney failure. Another possible cause of dehydration is the diuretic effect of MEL, which has been documented in dogs (Lipschitz and Stockey, 1945). Serum concentrations of ALB, TP, and GLOB can all be elevated during periods of dehydration (Cornell, 2010a; Nicoll et al., 2012). This concentration of solids in the blood, which is usually the result of fluid loss to the tissues, is termed hemoconcentration (Merriam-Webster, 2012). The occurrence of hemoconcentration has been reported during periods of dehydration (Diseases, 2008).

The increase in UA during the present study, in ducks fed ≥ 1.00 % MEL, can be caused by decreased renal function. Increased levels of UA are used to diagnose renal failure, with increased levels occurring when more than 70 % of kidney function is lost (Cornell, 2010c). Ducks that consumed 1.50 % MEL had UA levels that were 4.4 times higher than levels in control animals. Increases in UA levels has also been reported in mature laying ducks fed ≥ 50 ppm of MEL for 21 days (Gao et al., 2010) and young Pekin ducks fed ≥ 1.00 % MEL (Chapter 3).

The increases in serum ALB, TP, GLOB, and UA levels of ducks fed ≥ 1.00 % MEL confirms the findings during gross and histopathology that the kidneys of ducks fed

≥ 1.00 % MEL appeared pale and enlarged, with mild dilation of the embryonal nephrons and collecting tubules. These findings also confirm the cause of death, renal failure, which was seen in the ducks that died during the study.

The fact that ducks fed CYA (≤ 1.50 %) or MEL + CYA (≤ 1.50 % of each compound) did not have increases in any serum parameter measured (GLU, ALB, TP, GLOB, CA, AST, GGT, or UA) suggest that renal function was unaffected. Gross appearance of the kidneys during termination revealed no apparent changes in size or color among the kidney of ducks fed CYA (≤ 1.50 %), MEL + CYA (≤ 1.50 % of each compound) or controls. This is supported by the histopathology reports that CYA (≤ 1.50 %) or MEL + CYA (≤ 1.50 % of each compound) fed ducks had kidneys that appeared normal and unaffected by treatment.

The detection of MEL in the kidney, breast tissue, and bile observed in ducks fed MEL during the current study were also observed in a previous study that involved feeding graded levels of MEL to Pekin ducks (Chapter 3). The bile of ducks fed MEL alone had the highest average MEL residues values (383 ppm) followed by the kidney (154 ppm) and finally the breast muscle (93 ppm). The ability of the duck kidney to accumulate MEL has been document by (Yan et al., 2009). These data are in agreement with data presented in Chapter 3, where feeding graded levels of MEL (up to 2.25 %) to young Pekin ducks, resulted in the bile having the highest residue level, followed by the kidney and then breast muscle. These data suggest that ducks can use the hepatobiliary system along with the kidneys as ways to eliminate MEL from their body. The ability to eliminate MEL via the bile has been suggested to occur in ducks (Chapter 3) and in broilers and poults (Brand, 2011). After feeding graded levels of MEL (up to 0.1 % of

the diet) to broilers for 42 consecutive days Lu et al. (2009) reported that breast meat had lower MEL residue levels than the kidney.

In the current study, MEL + CYA (≤ 1.50 % of each compound) had lower residue values in the kidney, breast muscle, and bile than ducks fed ≤ 1.50 % MEL. Reimschuessel et al. (2008) observed similar results in fish, noting that fish that had received only MEL or CYA usually contained higher residue levels of the compounds as compared to fish that were administered both compounds simultaneously. Reimschuessel et al. (2008) went on to state that the decrease in muscle residues levels was due to the MEL-CYA complex precipitating out in the gastrointestinal tract and kidneys, thus decreasing its bioavailability. The results of the current study support this hypothesis by Reimschuessel et al. (2008), since lower muscle residue levels were detected in MEL + CYA (≤ 1.50 % of each compound) fed ducks as compared to ducks fed ≤ 1.50 % MEL. Ducks fed MEL + CYA (≤ 1.50 % of each compound) also had lower kidney residue levels than ducks fed MEL (≤ 1.50 %). It is possible that most of the precipitation of the MEL-CYA complex occurs in the gastrointestinal tract, thus not allowing MEL to be absorbed. Another possibility is that ducks, unlike fish, can eliminate MEL in the bile, thus decreasing the amount of MEL that passes through the kidneys.

With no lesions or pathologic changes observed in the liver section of ducks from all treatments it appears that MEL, CYA, or MEL + CYA at these dietary inclusion levels does not cause hepatic damage. Histopathology results also indicate that CYA, up to 1.50 % of the diet and fed for 21 consecutive days does not damage the kidney of young Pekin ducks. The addition of ≥ 1.00 % MEL does cause renal lesions that are compatible with mild MEL toxicity. Renal damage seen in ducks fed ≥ 1.00 % MEL contributed to

or directly caused the decreased performance and changes in blood serum chemistry documented in the current experiment. Similar results in the gross and histopathology appearance of the kidney have been documented in poults and broilers fed MEL (Brand, 2011). However, addition of CYA to a diet contaminated with MEL protected the kidney against the damaging effects of MEL. This ability of CYA to alleviate the negative effects of MEL has been documented in poults fed similar dietary levels of MEL and CYA (Brand, 2011). However, the combination of MEL and CYA was more toxic than MEL alone when fed to broilers at similar dietary levels as was used in the current study (Brand, 2011).

Microscopic examination of the bile also determined the presence of MEL crystals in ducks fed ≥ 1.00 % MEL. Crystals were not observed in the bile of ducks fed 0.50 % MEL, ≤ 1.50 % CYA, and MEL + CYA (up to 1.50 % of each compound). Figures 4.3 and 4.5 shows the contrast in the concentration of MEL crystals present in the bile of a duck that died during the study (Figure 4.3) to one that survived to termination (Figure 4.5) (Both ducks received 1.50 % MEL). This suggests a greater accumulation/concentration of MEL in the bile of the duck that died during the study. Therefore, this duck might have been more efficient in absorbing MEL or less efficient in excreting MEL. Differences in sensitivities to MEL was reported by Brand (2011) who observed that a high number of poults died (due to renal failure) within the first half of the experiment while others, consuming the same concentration of MEL in their diet, survived until termination. Others have reported that birds fed MEL for 42 days had lower tissue residue levels of MEL than birds that consumed MEL for 28 days (Lu et al., 2009). Lu et al. (2009) went on to hypothesize that as birds age they develop a better

capacity to clear MEL from their body. Another hypothesis is that as these ducks age residue values appear lower since the duck have increased in size, thus diluting the MEL that is deposited in their tissues.

In conclusion, with the documented changes in performance, serum chemistry values, and gross and microscopic appearance of the kidneys, the study indicates that ducks fed ≥ 1.00 % MEL had decreased renal function leading to decreased growth and eventually death. It is also evident that inclusion of up to 1.50 % CYA was not toxic to young Pekin ducks. These data also indicate that inclusion of CYA to diets that contain MEL (in a one to one ratio of MEL to CYA) is able to reduce the negative effect of MEL in young Pekin ducks. It appears this decrease in toxicity of MEL due to CYA is from the precipitation of the MEL-CYA complex in the gastrointestinal tract, thus decreasing the absorption of MEL as compared to ducks fed MEL alone (Reimschuessel et al., 2008). It also appears that young ducks can eliminate MEL via their bile, a process that has been suggested to occur in other avian species (Brand, 2011).

Table 4.1. Ingredients and nutrient composition of basal ration

Ingredient	Composition (%)
Corn	58.14
Soybean Meal	35.04
Dicalcium Phosphate	1.46
Limestone	0.51
Soybean Oil	1.06
Salt	0.33
Vitamin/mineral Mix[1]	0.35
DL-Methionine	0.11
Sand	3.00
Total	100.00
Nutrient composition (calculated)	
Crude Protein (%)	22.00
Metabolizable Energy (kcal/Kg)	2,900
Lysine (%)	1.19
Methionine (%)	0.44
Methionine + Cysteine (%)	0.80
Threonine (%)	0.82
Calcium (%)	0.65
None Phytate Phosphorus (% Av.)	0.40

[1]Vitamin/Mineral mix provided (mg/kg of diet): manganese, 40,000; zinc, 40,000; iron, 20,000; copper, 4,500; iodine, 600; selenium, 60; riboflavin, 2,640; pantothenic acid, 2,640; niacin, 11,000; vitamin B_{12}, 4.0; choline, 154,000; biotin, 13.2; folacin, 275; thiamin, 550.

Table 4.2. Individual and combined effects of melamine and cyanuric acid on performance and organ weights of Pekin ducks

Treatment[1]		Response Variables[2]					
Melamine (%)	Cyanuric Acid (%)	BWG (g)	FI (g)	F:G (g:g)	Mortality[3] (%)	Liver[4] (%)	Kidney[4] (%)
0.00	0.00	897^{ab}	1,857^{a}	2.07^{bc}	0^{c}	3.74	1.11^{c}
0.50	0.00	857^{ab}	1,783^{a}	2.09^{bc}	4^{bc}	4.01	1.26^{bc}
1.00	0.00	660^{c}	1,361^{b}	2.25^{b}	16^{b}	3.89	1.55^{b}
1.50	0.00	447^{d}	868^{c}	2.49^{a}	44^{a}	3.97	2.22^{a}
0.00	0.50	923^{a}	1,897^{a}	2.05^{c}	0^{c}	3.94	1.12^{c}
0.00	1.00	880^{ab}	1,759^{a}	2.00^{c}	0^{c}	4.06	1.11^{c}
0.00	1.50	870^{ab}	1,826^{a}	2.11^{bc}	0^{c}	3.90	1.04^{c}
0.50	0.50	871^{ab}	1,820^{a}	2.09^{bc}	0^{c}	4.02	1.14^{c}
1.00	1.00	839^{ab}	1,721^{a}	2.05^{c}	0^{c}	3.91	1.16^{c}
1.50	1.50	829^{b}	1,701^{a}	2.05^{c}	0^{c}	3.79	1.08^{c}
ANOVA:[5]	S.E.M.	28	68	0.06	0.1	0.19	0.12
	P-value	< 0.0001	< 0.0001	0.0002	< 0.0001	0.9778	< 0.0001

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

[2]Data are means of five replicate pens with five ducks per pen for control and treatments fed melamine alone or melamine + cyanuric acid. Data are means of four replicate pens with four ducks per pen for treatments fed cyanuric acid alone.

[3]Means are percents of mortality that occurred out total number of ducks in each treatment. 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.

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

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

Table 4.3. Individual and combined effects of melamine and cyanuric acid on serum chemistry of Pekin ducks

Treatment[1]		Response Variables[2]							
Melamine (%)	Cyanuric Acid (%)	GLU (mg/dL)	ALB (g/dL)	TP (g/dL)	GLOB (g/dL)	CA (mg/dL)	AST (U/L)	GGT (U/L)	UA (mg/dL)
0.00	0.00	223	1.31^{c}	3.18^{cd}	1.87^{cd}	11.69	62.7	3.6	5.75^{c}
0.50	0.00	188	1.38^{bc}	3.39^{bc}	2.01^{bc}	12.14	21.1	3.9	7.95^{c}
1.00	0.00	223	1.50^{ab}	3.68^{ab}	2.18^{b}	11.63	55.2	4.1	15.56^{b}
1.50	0.00	349	1.55^{a}	3.96^{a}	2.41^{a}	12.34	94.2	3.6	25.48^{a}
0.00	0.50	291	1.28^{c}	3.17^{cd}	1.89^{cd}	11.14	59.5	3.4	8.38^{c}
0.00	1.00	254	1.26^{c}	2.98^{d}	1.72^{d}	12.03	59.2	2.8	6.11^{c}
0.00	1.50	208	1.28^{c}	3.05^{d}	1.77^{d}	11.79	34.7	3.3	4.83^{c}
0.50	0.50	222	1.34^{c}	3.22^{cd}	1.88^{cd}	11.88	59.9	3.1	5.45^{c}
1.00	1.00	271	1.27^{c}	3.07^{d}	1.80^{d}	11.69	17.7	3.9	4.70^{c}
1.50	1.50	269	1.25^{c}	3.03^{d}	1.78^{d}	11.78	24.4	3.4	6.13^{c}
ANOVA:[3]	S.E.M.	38	0.05	0.11	0.06	0.41	25.95	0.3	2.35
	P-value	0.1671	0.0004	< 0.0001	< 0.0001	0.7603	0.5731	0.2163	< 0.0001

[1]Treatments were the addition of melamine and or cyanuric acid, in percent indicated, to a basal diet. Diets were fed for 21 consecutive days.
[2]Data are means of ten ducks per treatment.
[3]One way analysis of variance values.
[a-d]Means within a column with no common superscript are different ($P < 0.05$).
GLU = glucose; ALB = albumin; TP = total protein; GLOB = globulin; CA = calcium; AST = asparate transaminase; GGT = gamma glutamyltranserase; UA = uric acid

Table 4.4. Residue levels of melamine in kidney, breast muscle, and bile of Pekin ducks

Treatment[1]		Response Variables		
Melamine (%)	Cyanuric Acid (%)	Kidney[2] (ppm)	Breast Muscle[2] (ppm)	Bile[3] (ppm)
0.00	0.00	ND[c]	ND[d]	ND
0.50	0.00	46[b]	32[b]	92
1.00	0.00	179[a]	93[ab]	490
1.50	0.00	238[a]	155[a]	568
0.00	0.50	ND[c]	ND[d]	ND
0.00	1.00	ND[c]	ND[d]	ND
0.00	1.50	ND[c]	ND[d]	ND
0.50	0.50	13[b]	11[c]	23
1.00	1.00	12[b]	ND[d]	30
1.50	1.50	ND[c]	ND[d]	24
ANOVA[4]	S.E.M.	2.1	2.1	-
	P-value	< 0.0001	< 0.0001	-

[1]Treatments were the addition of melamine and or cyanuric acid, 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 was performed on transformed data (ranked data).
[3]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.
[4]One way analysis of variance values.
[a-d] Means within a column with no common superscript are different ($P < 0.05$).
ND = none detected.

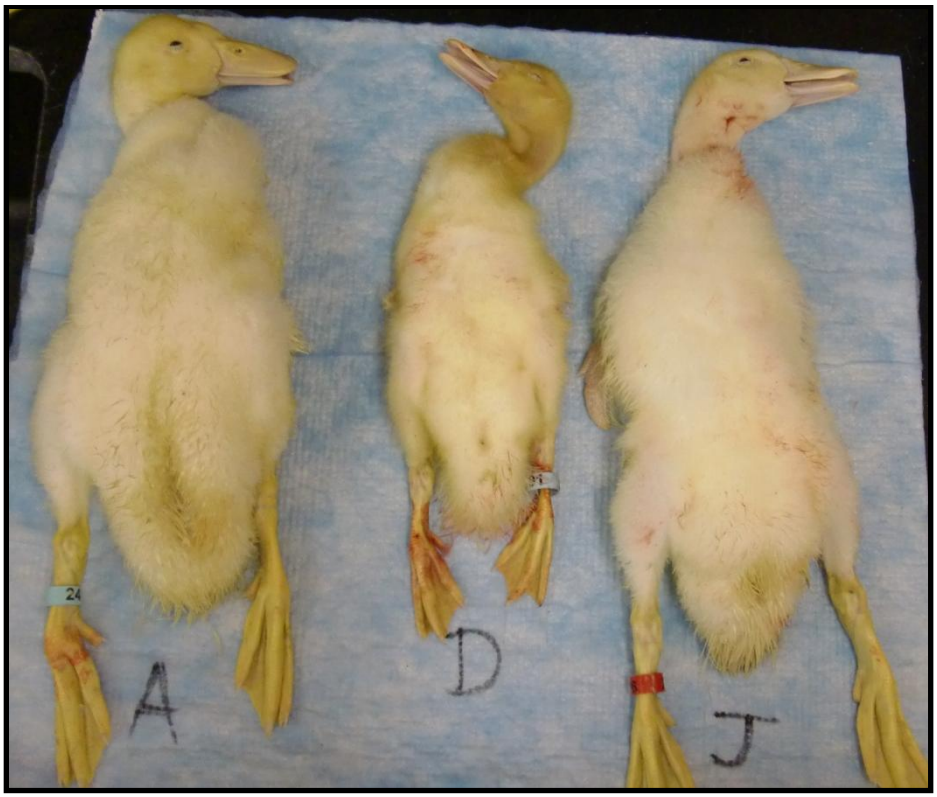


Figure 4.1. Effects of melamine and/or cyanuric acid on the performance of young Pekin ducks fed treatments from three days of age to 21 days of age. Duck on left (A) belongs to the control treatment while the duck in the middle (D) received a diet containing 1.5% MEL. The duck on the right (J) received a diet containing 1.50 % MEL + 1.50 % CYA.

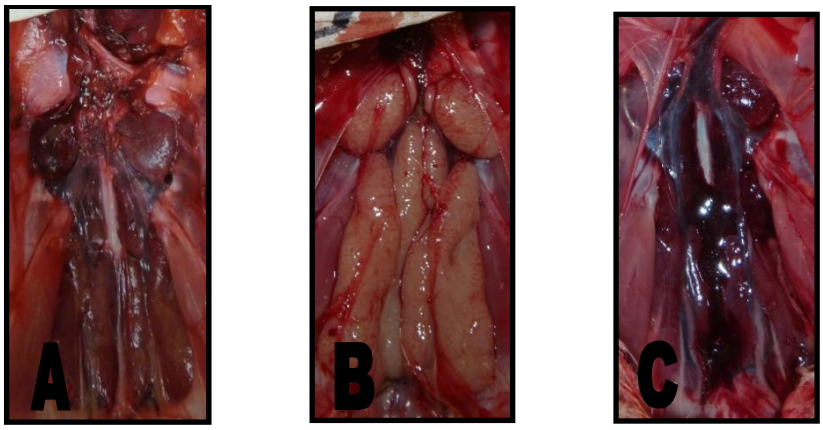


Figure 4.2. Effect of melamine on the kidneys of young Pekin ducks fed treatments from three days of age to 21 days of age. A) Kidney of control fed duck. B) Enlarged and pale kidney of duck fed control diet + MEL (1.50 %). C) Kidney of duck fed MEL + CYA (1.50 % of each compound).

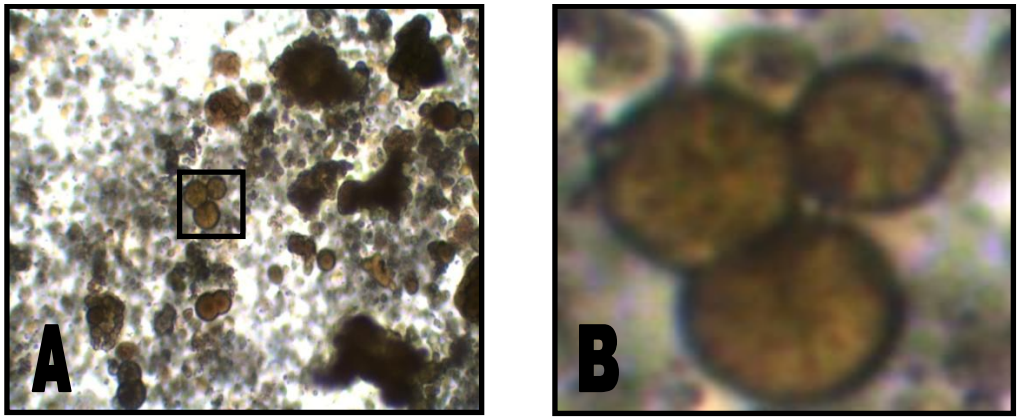


Figure 4.3. Crystals in the bile of early mortality ducks fed 1.50 % melamine. Photo 'A' is taken at 100 x magnification. The Expanded view shows three round crystals that appear brown in appearance. Photo 'B' is an expanded view of three crystals that are shown in photo 'A'.

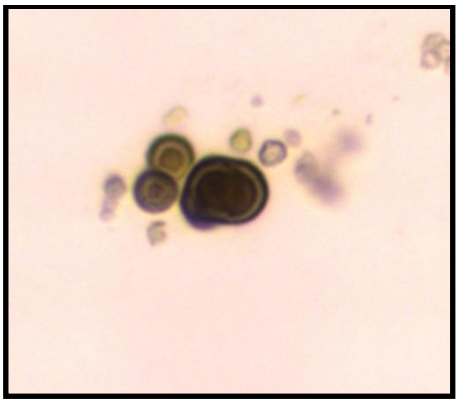


Figure 4.4. Crystals in the bile of ducks fed 1.00 % melamine and survived to termination. Photo taken at 200 x magnification, shows round, brown crystals in the bile of ducks fed.

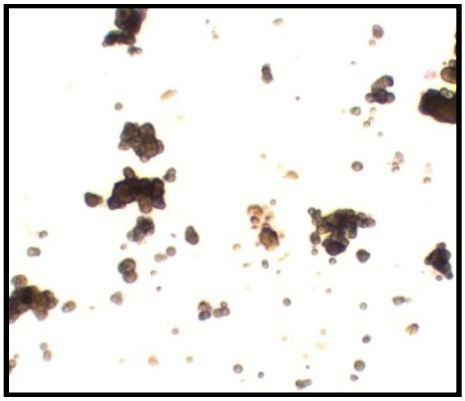


Figure 4.5. Crystals in the bile of ducks fed 1.50 % melamine that survived to termination. Photo taken at 100 x magnification.

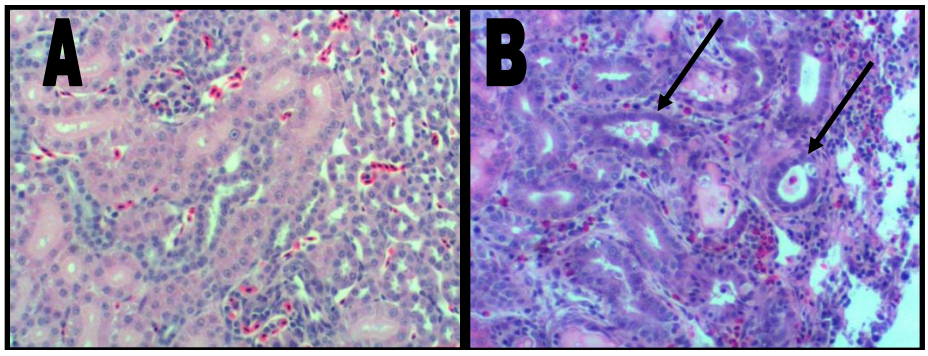


Figure 4.6. Kidney sections from a control duck and one fed 1.50 % melamine. Photo 'A' is a kidney section from a control duck viewed at 100 x magnification. Photo 'B' is a kidney section from a duck fed 1.50 % MEL and viewed at 100 x magnification. Note dilated collecting tubules with casts present in photo 'B'.

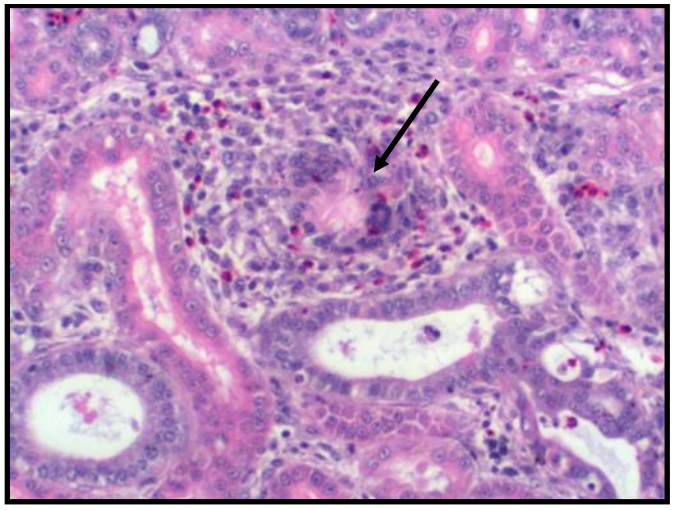


Figure 4.7. Kidney section from a duck fed 1.50 % melamine. Viewed at 100 x magnification casts and crystals are present in the interstitial space.

Chapter 5

Effects of Melamine in Young Barrows

Introduction

Melamine ($C_3H_6N_6$) is a white, crystalline powder (OSHA, 2006) with a wide variety of industrial applications including use in manufacturing of plastics, adhesives, laminates, paints, flame retardants, textiles finishes, and fertilizers (Hilts and Pelletier, 2009). Cyanuric acid, along with ammeline and ammelide, are structural analogues of melamine (Tyan et al., 2009; WHO, 2008a) and all belong to the class of chemicals known as *s-triazines* (Wackett et al., 2002). The ability of melamine and cyanuric acid to self-assemble and form a hydrogen-bonded bimolecular network (Perdigao et al., 2006), allows the compounds to precipitate in the kidneys of animals that have ingested them, causing renal failure (Seffernick et al., 2010).

Contamination of feed or food with melamine and/or cyanuric acid can occur indirectly and accidently by treatment of feed ingredients with products that contain melamine or cyanuric acid (WHO, 2008a). However, in recent years the intentional adulteration of feed and food with melamine (Hilts and Pelletier, 2009) has received international attention. Melamine is 66 % nitrogen (Yang et al., 2009), therefore, protein analysis using the Kjeldahl method, will result in an invalid or an over estimate of the actual protein content of a matrix that contains melamine (Yang et al., 2009). For this

reason melamine was intentional added to feed ingredients or feeds to increase their monetary value (Cianciolo et al., 2008).

Precipitation of melamine-cyanuric acid crystals in the kidneys of dogs and/or cats was documented by Brown et al. (2007), Cianciolo et al. (2008), and Puschner et al. (2007). It has also been well documented (Brown et al., 2007; Dobson et al., 2008) that blood urea nitrogen (BUN) levels increase in animals fed melamine and cyanuric acid due to a decrease in kidney function. Studies by Tolleson (2009) and Dobson et al. (2008) describe possible routes of absorption of melamine and related compounds as well as how precipitation of the compounds can lead to renal damage and renal failure.

In 2007, renal failure, caused by ingestion of pet food that was contaminated with melamine and cyanuric acid, caused the death of pets across North America and South Africa (Hilts and Pelletier, 2009). Soon after, it was determined that pet food scraps containing melamine and cyanuric acid were incorporated in swine, poultry, and aquaculture feeds (Buur et al., 2008; Karbiwnyk et al., 2009; USDA, 2007a). Animals that consumed the contaminated feed were placed under quarantine by the USDA Food Safety and Inspection Service (FSIS) until it was certain that meats and other products from these animals were not themselves contaminated (USDA, 2007a).

In September 2008, reports from China indicated that 52,875 children had been treated for renal complications after having consumed infant formula and other related dairy products contaminated with melamine (Chan et al., 2008; WHO, 2008a). By November 2008, some 294,000 infants in China had been affected, with 50,000 hospitalized, and six reported deaths (Chan et al., 2008; WHO, 2008a). It was determined that melamine was used to artificially increase the crude protein of milk that

was used in the production of infant formula (PEHSU, 2009; WHO, 2008a). However, unlike the pet food incidence of 2007, the melamine added to the milk was relatively pure, with levels of cyanuric acid, ammeline, and ammelide about 0.1% that of the melamine level (WHO, 2008a). This level of contamination with cyanuric acid, ammeline, and ammelide, was much lower than the levels present in the pet food products in 2007 (WHO, 2008a).

Since the 2007 and 2008 incidents of contamination of animal feed and human food with melamine and related analogs several case reports and experiments have been published involving consumption of melamine and related compounds by swine. Gonzalez et al. (2009), Lee et al. (2011), and Nilubol et al. (2009) each documented cases where melamine and or related analogs were incorporated into swine feed. Nilubol et al. (2009) reported that starter diets fed to swine on a farm in Thailand had melamine, cyanuric acid, and ammeline concentrations of 3,209 ppm (0.32 % of the diet), 1,126 ppm (0.11 % of the diet), and 949 ppm (0.09 % of the diet), respectively. Mortality started approximately two weeks after weaning and within two months approached 100%. Kidneys from affected pigs appeared yellowish, slightly swollen, glistening, and showed perirenal edema. Crystal precipitates were evident on the surface of cut renal sections, with crystals appearing yellow to brown with radiating striations (Nilubol et al., 2009). Lee et al. (2011) documented melamine concentrations that ranged from 750 ppm (0.075 % of the diet) to 1,500 ppm (0.15 % of the diet) in a swine diet in Malaysia. During consumption of the contaminated feed Lee et al. (2011) reported pigs appearing inappetant, anorexic, thin, dull, and depressed. The kidneys from these pigs were enlarged and yellowish in appearance. Severely affected pigs had kidneys that were

small in size with clear pitting and dimpling, dark red and brown in color, with multiple cysts present in the cortex.

Reimschuessel et al. (2008) provided the first experimental data regarding the consumption of melamine by swine. In his research, Reimschuessel et al. (2008) fed 400 mg/kg BW (0.7 % of the diet) of melamine and cyanuric acid, alone or in combination, to 16 week old Yorkshire-cross pigs. A three day feeding period was used with one pig per treatment. Pigs fed melamine alone and cycauric acid alone showed no gross lesions or crystals in the kidneys, and had normal blood urea nitrogen and creatinine levels. Only the pigs fed the combination diet developed golden-brown crystals that formed radial spheroid aggregates in the kidneys. The combination fed pigs also had elevated blood urea nitrogen and creatinine levels. Reimschuessel et al. (2008) also suggested that melamine-cyanurate and melamine-uric acid crystals might not be stable in formalin and would dissolve when tissues are fixed in the formalin.

In 2011, a no-observed-adverse-effect-level (NOAEL), was determined in pigs by Stine et al. (2011). Three separate studies were conducted with weanling barrows fed melamine or cyanuric acid alone or in combination. The first study, a preliminary study, found a dose related response in kidney weight and relative kidney weight when melamine-caynuric acid was fed at levels greater than 10 mg/kg BW/day (0.025 % of the diet). Crystalline structures were found in the medulla and cortex of kidneys from barrows fed 10 mg/kg BW/day or more of the melamine-cyanuric acid combinations, and in barrows fed 200 mg/kg BW/day (0.50 % of the diet) of melamine alone. In the second experiment Stine et al. (2011) fed 0.0, 1.0, and 3.3 mg/kg BW/day (0.0, 0.0025, and 0.00825% of the diet) of melamine-cyanuric acid to barrows for 28 days. Only one

barrow fed 3.3 mg/kg BW/day developed a cluster of crystals in the kidneys, therefore the NOAEL was set at 1.0 mg/kg BW/day of melamine in combination with 1.0 mg/kg BW/day cyanuric acid. Finally, a follow-up study was conducted by feeding 200 mg/kg BW/day of melamine to barrows. Upon examination no crystals were present in pigs feed 200 mg/kg BW/day.

During the experiments by Stine et al. (2011), ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) was used to determine crystal composition. It was found that crystals removed from the kidney of pigs fed melamine only were closely related to the morphology of crystals from pigs fed combinations of melamine and cyanuric acid. Similar morphology and a one to one ratio of melamine to cyanuric acid, determined by UPLC-MS/MS, indicates that crystals from the melamine fed pig contained both melamine and cyanuric acid (Stine et al., 2011). Stine et al. (2011) stated that while microbial conversion of melamine to cyanuric acid, documented by Seffernick et al. (2010) and Wackett et al. (2002), does occur, it is unclear if the microorganisms responsible for the conversion reside and are active in the gastrointestinal tract of the animal.

Previous research has determined that combinations of melamine and cyanruic acid can form renal crystals similar to those detected in pets (Reimschuessel et al., 2008) and established a NOAEL for pigs fed a combination of melamine and cyanuric acid (Stine et al., 2011). However to date, no research has addressed the effects of higher dietary levels of melamine in young weanling pigs or tissue residue levels after consumption. Therefore, the objectives of the current study were to determine the effects

of high dietary levels of melamine (up to 1.25 % of the diet) on weanling barrows, and to determine melamine residue levels in the kidney, muscle, and bile.

Materials and Methods

Diet Preparation:

A basal diet (Table 5.1) was formulated to meet or exceed all requirements of weanling pigs set forth by the National Research Council (NRC, 1998). Six dietary treatments were prepared from the basal diet by adding melamine (MEL), purchased from Fisher Scientific. MEL was substituted for sand to obtain the desired dietary MEL concentrations (0.00, 0.25, 0.50, 0.75, 1.00, and 1.25 %).

Pigs, Management, and Response Variables:

The animal care and use protocol was reviewed and approved by the University of Missouri Animal Care and Use Committee (ACUC). Thirty barrows, two weeks post weaned, were purchased from a commercial producer, weighed, ear tagged, and placed in individual pens with *ad libitum* access to feed and water. A completely randomized design was used, with five replicate pens of one pig each assigned to each of the six dietary treatments. Pigs were housed in an environmentally controlled building with elevated 1.2 m^2 pens with plastic covered grate flooring over a flush system. Each pen had a stainless steel nipple waterer and a three-hole nursery feeder. Dietary treatments were fed for 21 days with each pig inspected daily for signs of illness.

On day 21, two blood samples per pig were taken from pigs in the first three replicates. The blood was collected via the anterior vena cava. Samples were collected into a 20 mL vacutube, one containing heparin and one without heparin. A 20 gauge

(2.54 cm) needle for hematology was used for collection. Both samples were sent to the University of Missouri Veterinary Diagnostic Laboratory (Columbia, MO) for analysis. Samples collected in tubes containing heparin were used for a complete blood count (CBC), which involves analysis of white blood cells, (WBC), red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and platelets (PLT). Blood samples collected in tubes without heparin were centrifuged (Sorvall, RC 3 B plus) at 1,400 x g for 30 min at 7ºC before serum was separated. Serum was analyzed for glucose (GLU), blood urea nitrogen (BUN), creatinine (CREA), sodium (Na), potassium (K), chloride (Cl), albumin (ALB), total protein (TP), globulin (Glob), calcium (Ca), phosphorus (Phos), magnesium (Mg), total bilirubin (T Bil), direct bilirubin (D Bili), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), and creatine phosphokinase (CPK). The following day (day 22) blood samples from the remaining two replicates were taken and treated in the same manner as the first group. Pigs were bleed and later killed in two groups due to time restrictions on the necropsy floor.

On day 21, all pigs and feed were weighed and average body weight gain, average feed intake, and feed conversion were calculated. On day 22, pigs in the first three replicates were euthanized via captive bolt followed by exsanguination, after which necropsies were performed at the University of Missouri Veterinary Diagnostic Laboratory (Columbia, MO). The remaining pigs in the final two replicates were euthanized in the same manner as the first group on day 23 of the study. The pigs were bled and killed in two groups due to time constraints on the kill floor.

During necropsies the liver and kidneys were removed from each pig and weighed to calculate relative weights (organ weight/body weight). Sections of liver, kidney, and muscle (hind quarter) were taken from each pig and fixed in 10 % neutral buffered formalin for histopathology. For histopathology tissues were also fixed in a 70 % ethanol solution for 24 h, after which they were placed in a 95 % ethanol until the tissues were trimmed for tissue processing and saved. Additional samples of kidney and muscle (hind quarter) along with bile were frozen for later analysis of MEL residue by high pressure liquid chromatography (HPLC).

Urine was collected from two pigs per treatment and sent to the University of Missouri Veterinary Diagnostic Laboratory (Columbia, MO) for a complete and physical/chemical urinalysis (color, clarity, specific gravity, glucose, bilirubin, ketones, heme, pH, protein dipstick, SSA, urobilinogen, presence of white blood cells, red blood cells, bacteria, round cells, squamous cells, casts, and crystals).

Two extra pigs were purchased to replace early mortalities and to ensure only high quality pigs were used in the experiment. These pigs were fed the same basal diet as pigs in the experiment. However, on day three of the experiment, since no mortality occurred, the extra pigs were given a basal diet (same basal diet used to formulate experimental treatments) that contained a combination of 0.75 % MEL and 0.75 % cyanuric acid (CYA) (Fisher Scientific). These pigs were treated the same as the experimental pigs. Feed intake, body weight gain, and all tissue collection and analysis were performed in the same manner as the experimental pigs. The purpose of feeding the combination diet to the extra pigs was to obtain kidney crystals (MEL-CYA) to compare to crystals that might occur in the MEL only fed pigs. Extra pigs (MEL + CYA) are not

included in any statistical analysis and are not included when treatment group is used to describe effects of MEL on barrows.

Melamine Analysis:

MEL extraction from tissue and bile samples was based on the method used by Brand (2011), and involved high-performance liquid chromatography (HPLC) via UV detection. For kidney and muscle, 10 mL of water:acetonitrile (1:2) was added to 1 g of tissue and the tissues homogenized 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; Sparks, MD) and the supernatant transferred to microcentrifuge tubes and further centrifuged for 5 min at 10,000 rpm (Spectrafuge 16M; Woodbridge, NJ). The supernatant was extracted and filtered through a MycoSep® 224 AflaZon columns (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 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 d min at 10,000 rpm (Spectrafuge 16M). The supernatant was collected and filtered through MycoSep® 224 AflaZon column (Romer Labs, 2011). Finally, 500 µL of the filtered supernatant 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 fluorescence detector, Hitachi Model L-7200 autosampler with Hitachi D-7000 data acquisition interface and ConcertChrom software on a microcomputer were used for HPLC analysis (Tokyo,

Japan). A HyperClone (Phenomonex) C_{18} column (100 x 4.60 mn) was used with a retention time of 6 min and flow rate of 1 mL/min. UV detection occurred at 240 nm. The mobile phase consisted of BUFF:acetrilnitril(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 curve was calculated, plotted on the standard curve, and used to calculate individual MEL concentrations in samples. The limit of detection was set at 8.0 ppm.

Statistical Analysis:

Data were analyzed using the general linear model procedures of Statistical Analysis Software (SAS) (SAS, 2006). Pen was 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 ≤ 0.05. A $\log_{10}$ transformation was applied to kidney MEL residue data before statistical analysis was performed. Data for MEL residue levels in the muscle was ranked by a method developed by Conover and Iman (1981) before analysis was performed. Regressions analysis was not performed on ranked data.

Results

Performance and Mortality:

The effects of MEL on body weight gain, feed intake, feed conversion and mortality are shown in Table 5.2. No mortality occurred during the 21 day treatment period for MEL or MEL + CYA fed pigs. Body weight gain and average daily gain decreased linearly ($P = 0.0005$) as percent MEL in the diet increased. Inclusion of ≥ 1.00 % MEL in the diet caused body weight gain and average daily gain to be less than ($P = 0.0205$) that of controls. Figure 5.1 shows the difference in pig size at the end of the 21 day experiment. The back pig (tag 18) in Figure 5.1 is a control pig which gained 12.76 kg during the 21 day study. The front pig in Figure 5.1 (tag 28) is a pig which received 1.25 % MEL in the diet, and gained 9.44 kg. Pigs fed MEL + CYA had an 18 day gain of 7.76 kg.

Gain to feed decreased in a quadratic ($P = 0.0407$) fashion with linear ($P = 0.0076$) sections as MEL levels in the diet increased. Pigs receiving 1.2 5 % MEL had a lower ($P = 0.0427$) gain to feed value than controls. Gain to feed of MEL + CYA fed pigs was 0.51. Feed intake was not affected ($P = 0.7769$) by the addition of up to 1.25 % MEL in the diet. However, pigs fed MEL + CYA consumed 26 % less feed than controls and 15 % less feed that pigs fed 1.25 % MEL.

Organ Weights:

Table 5.2 shows the effects of dietary inclusion of MEL up to 1.25 % of the diet, on organ weights of young barrows. Liver weight, kidney weight, relative liver weight (% of body weight), and relative kidney weight (% of body weight) were not affected ($P > 0.05$) by increasing dietary levels of MEL. Figure 5.2 shows that macroscopically the

liver and kidneys from a control fed pig (tag 24) and pig fed 1.25 % MEL (tag 15) were similar.

The similarity of organ appearance among control pigs and pigs fed MEL alone is in sharp contrast to Figure 5.3 which depicts the kidneys from a control pig (tag 26) and a pig fed MEL + CYA (tag 2). The kidney from the MEL + CYA fed pig is larger in appearance and yellow in color than the kidney from the control pig.

Complete Blood Count (CBC):

The results of the CBC are summarized in Table 5.3. Statistically there were no treatment differences ($P > 0.05$) for WBC, RBC, HGB, HCT, MCHC or PLT. MCH decreased in a linear ($P = 0.0393$) fashion with increasing dietary concentrations of MEL. MCV was significantly lower from control levels, in pigs fed 1.25 % MEL. This decrease in MCV as dietary MEL levels increased was determined to be linear ($P = 0.0068$). Pigs fed MEL + CYA had WBC counts that were 66 % greater than that of control pigs and 60 % greater than that of pigs fed 1.25 % MEL.

Serum Chemistry:

The treatment means for the large animal maxi profile are summarized in Table 5.4 and Table 5.5. A linear decrease ($P = 0.0124$) was also observed for serum GLU as dietary MEL levels increased. In contrast, AST increased ($P = 0.0049$) in a linear fashion as MEL inclusion in the diet increased. MEL + CYA fed pigs had serum levels of BUN that was 11 fold greater than control pigs and 12 fold greater that pigs fed 1.00 % MEL. MEL + CYA fed pigs had a mean CREAT level that was 7 fold greater than that of control pigs and pigs fed 1.25 % MEL. There was a linear ($P = 0.0053$) decrease in serum Ca levels as percent MEL in the diet increased.

Urinalysis:

Urine analysis from two pigs per treatment showed no difference in any of the parameters measured (clarity, specific gravity, glucose, bilrubin, ketones, heme, pH, protein dipstick (4+ scale), urobilinogen, WBC, RBC, bacteria, round and squamous cells, and casts). However, one pig each from the control, 0.50 % MEL, 0.75 % MEL, and 1.00 % MEL groups presented with amorphous crystals. One pig each from the 1.00 % MEL and 1.25 % MEL groups presented with brushite crystals present in the urine. One pig fed 1.25% MEL presented with MEL crystals. Figure 5.4 shows the similarity between crystals taken from the urine of pigs fed 1.25 % MEL and MEL + CYA (photos 'E' and 'F', respectively). These crystals are similar in structure to crystals found by Stine et al. (2011), who fed 100 mg/ kg MEL + 100 mg/kg CYA to young barrows (Figure 5.4. photo 'G').

Additional analysis of urine from one pig of the control, 1.25 % MEL and MEL + CYA groups was performed. This revealed calcium oxalate crystals present in the urine of the control pig and calcium phosphate crystals in the urine of the pig fed 1.25 % MEL. Crystals, with the structural appearance of MEL, were also found in the urine of the pig fed 1.25 % MEL and the pig fed MEL + CYA. Figure 5.4 shows each type of crystal present from the current study, along with examples of crystals of known origin for comparison. The calcium oxalate crystals are of little clinical value as they often develop if a urine specimen is left standing (Spencer, 2007). Calcium phosphate crystals, also called brushite, can indicate urinary tract infections but otherwise are of little clinical value (Spencer, 2007). The calcium phosphate crystals observed were probably the same crystal observed in the original urine analysis.

The MEL + CYA pigs both presented with white blood cells present in the urine. The urine also contained a moderate amount of crystals that were consistent with MEL-CYA complexes. All other parameters measured were similar to results obtained from treatment fed pigs.

Tissue Residue:

MEL levels in the kidney, muscle (hind quarter), and bile are summarized in Table 5.6. MEL levels in the kidney increased in a quadratic fashion ($P < 0.0001$) with linear components ($P < 0.0001$). Kidneys from pigs fed diets containing ≥ 0.25% MEL had residue levels greater than controls ($P < 0.0001$). MEL concentrations in bile increased linearly ($P < 0.0001$) with increasing MEL concentrations. Muscle ($P < 0.0001$) and bile ($P < 0.0001$) from pigs fed diet containing ≥ 0.50%MEL had residue levels higher than controls. MEL + CYA pigs had average MEL concentration of 32 ppm in the kidney, 17 ppm in the muscle and 46 ppm in the bile.

Pathology:

Gross Pathology:

No mortality occurred during the 21 day experimental period (Table 5.2.) including the MEL + CYA pigs. During termination, no differences were noted in liver and kidney size, or color among treatments. Figure 5.2 shows similar appearance in color and size of the kidneys and liver of a control pig (tag 24) and pig fed 1.25 % MEL (tag 15). No crystals were present when the bile of pigs fed 1.25 % MEL was viewed microscopically.

Livers from MEL + CYA pigs appear similar to that of livers from the control pigs. However, kidneys from the MEL + CYA pigs were 82 % larger than kidneys from

control pigs. Kidneys from MEL + CYA pigs were yellow in color. Figure 5.3 demonstrates the sharp contrast in size and color of kidneys from a MEL + CYA fed pig (tag 2) and a control pig (tag 26). Figure 5.5 shows the cross section of a kidney of a MEL + CYA fed pig. Note that crystals are clearly present to the naked eye around the renal medulla.

Histopathology:

Microscopic evaluation of liver, kidney, and muscle sections revealed no change in appearance among control pigs and pigs fed MEL alone. Figure 5.6 shows a microscopic section of a kidney from a control pig (photo A) and a pig fed 1.25 % MEL (photo B). No changes in appearance of kidney sections are apparent in Figure 5.6. Bile from a control pig, 1.25 % MEL fed pig, and MEL + CYA fed pig were examined microscropically and all were found to be free of crystals. However, three pigs, one from the 1.00 % MEL group and two from the 1.25 % MEL group, presented with moderate to severe inflammation in the liver, kidney, and muscle. The hepatitis was chronic (portal and periportal cholangiohepatitis) in general, with one pig having acute hepatitis. The hepatitis did not appear to be directly related to MEL consumption.

Histopathology of tissues from the MEL + CYA pigs revealed no microscropic lesions of the liver. The kidneys however, contained several lesions and crystals were noted during histopathological examination. Crystals were golden brown to yellowish in color and appeared round with spokes emanating from the center. Figure 5.7 shows several examples of crystals present in the kidney of MEL + CYA pigs. Photo 'A' from Figure 5.7 shows crystals spread throughout the kidney tissue. Photo 'B' shows the round appearance of the crystal with spokes emanating from the center. The final two

photos, ‘C’ and ‘D’, were taken under polarized light, the crystals, clearly visible, shine against the background.

It was also determined that while tissues fixed in formalin and ethanol had similar microscopic characteristics, the ethanol fixations preserved crystals slightly better. The total number of crystals in tissues fixed in formalin or ethanol was similar. However, crystals in the formalin fixed tissues had a tendency to break off leaving empty spaces in the tissue.

Discussion

Weight gain and gain to feed were the only performance variables that were significantly lower than controls when MEL levels were ≥ 1.00 % and equal to 1.25 % of the pig’s diet, respectively. Barrows fed 1.00 % and 1.25 % MEL gained 3.26 and 3.32 kg less than controls, respectively. While this decrease in weight gain for the 1.00 and 1.25 % MEL treatment groups was significant, no mortality occurred and feed intake, while numerically lower in groups fed MEL, was not significantly decreased. This suggests that while MEL consumed at ≥ to 1.00 % of the diet is not fatal, performance of barrows is reduced. These data confirms research by Stine et al. (2011), in which feeding 200 mg/kg BW/day (0.50 % of the diet) to barrows had no effect on daily gain. Similar to the current study Brand (2011) observed decreases in feed intake and body weight gain of young broilers when MEL was fed at levels ≥ 1.0 % of the diet. Body weight gain was reduced in poults fed 1.5 % MEL (Brand, 2011). Finally ducks fed ≥ 1.00 % MEL had decreased weight gain and feed intake (Chapter 3).

Blood urea nitrogen (BUN) is a measure of urea or the end product of protein metabolism and is excreted by the kidneys (Nicoll et al., 2012). Therefore, BUN is directly related to protein intake, nitrogen metabolism, and kidney excretion rate and can be used to measure kidney function (Nicoll et al., 2012). Creatinine (CREAT), a waste product generated from muscle metabolism (Nabili, 2012) is cleared from the body via filtration through the glomerulus (Nicoll et al., 2012). CREAT levels in the serum can be used to measure glomerular filtration rate, with each 50 % reduction in glomerular filtration rate correlating to a doubling of CREAT levels in the serum (Nicoll et al., 2012). In the current study, BUN and CREAT concentrations of pigs fed MEL alone were not different from controls and values were within or near (± 0.04) references ranges (Merck, 2011). This suggests, along with no changes in kidney size (kidney weight or relative kidney weight) and no changes in the appearance of the kidney (gross or microscopic), that MEL at levels ≤ to 1.25% of the diet does not affect the kidney function of young barrows.

Reimschuessel et al. (2008) reported similar results when feeding 400 mg/kg BW (0.7 % of the diet) of MEL to 16 week old pigs with no gross lesions observed in the kidney, and BUN and CREAT levels within the normal range. Stine et al. (2011), also reported that barrows fed 200 mg/kg BW/day MEL (0.5 % of the diet) for 28 days showed no differences among treatments in any variable measured (body weight gain, average relative kidney weight, BUN or CREAT). Only one of ten pigs from the Stine et al. (2011) study that received MEL had crystals present in the kidneys. The kidneys from the other nine pigs were unaffected by inclusion of MEL. Melnick et al. (1984) fed up to 18,000 ppm (1.8 % of the diet) MEL to rats for 13 weeks and noted that the kidneys

of male rats were unaffected by MEL. Brand (2011) reported that slightly higher levels of MEL (1.5 %) than was used in the current study caused mild accumulation of spherical basophilic crystals, and ≥ 2. 5 % MEL caused increased toxicity in young broilers. In poults, Brand (2011) observed a greater sensitivity to MEL, with ≥ 1.00 % MEL causing gross pathology that was associated with renal failure and ≥ 2.0 % MEL causing > 63 % mortality in poults.

Urine analysis revealed no difference among any variables measured. Only the presence of brushite (calcium phosphate), calcium oxalate, and amorphous crystals were different among treatments. The amorphous crystals identified from the current analysis are most likely amorphous urates or amorphous phosphate crystals. However, the presence of calcium phosphate and oxalate, and amorphous (urates or phosphate) crystals does not appear to be treatment related and are often of little clinical value (Spencer, 2007) . One pig fed 1.25 % MEL had crystals in the urine that appear similar to crystals taken from pigs fed MEL + CYA in the current study and from pigs fed MEL + CYA by Stine et al. (2011). Stine et al. (2011) analyzed the crystals from the MEL only fed pigs and found the crystals had MEL–CYA ratios of 1:1. With the ability of microorganisms to convert MEL to CYA documented (Jutzi et al., 1982; Wackett et al., 2002), more research is needed to confirm if this conversion of MEL to CYA can be accomplished by microorganisms that reside in the gastrointestinal tract of mammals (Stine et al., 2011) or alternatively if MEL can be metabolized to CYA by mammals.

Mean corpuscular volume (MCV), which measures the average volume of red blood cells (Nicoll et al., 2012), showed significant difference among treatments. However, MCV values from all treatments fell within the normal range for swine (Merck,

2011). Mean corpuscular hemoglobin (MCH) which indicates the amount of hemoglobin per red blood cell (Nicoll et al., 2012) decreased in a linear fashion as dietary MEL levels increased but all values were close to reference ranges (± 0.68) for swine (Merck, 2011). Serum aspartate aminiotransferase (AST) increased in a linear fashion as MEL levels increased. However, AST values from all treatments fell within the normal range for swine (Merck, 2011)

Blood serum Ca levels decreased linearly as dietary MEL levels increased. It is worth noting that decreased serum calcium levels can indicate renal insufficiency (Nicoll et al., 2012), although renal function in barrows from the current study appeared unaffected since BUN and CREAT levels were normal and unchanged by the treatment.

Linear decreases in serum GLU and ALB (numerical) were observed as MEL levels increased in the diet. The decrease in serum GLU and ALB values could potentially be explained by the numeric reduction in feed intake.

The highest MEL concentrations were found in the kidney and bile. MEL concentrations in the muscle were about half of what was found in the kidney and bile. For example, kidneys from 1.25% MEL pigs had MEL concentrations of 125 ppm, while the muscle only had 57 ppm MEL. Higher concentrations of MEL in the kidney are consistent with reports by Dobson et al. (2008) and Puschner et al. (2007) who noted precipitation of MEL and CYA complexes in the kidney probably due to an increase in the 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 increase, above concentrations in other tissue, due to the compound becoming more concentrated as the kidneys filter unwanted compounds (MEL) out of the blood and

into the urine. The fact that the bile had similar MEL values as the kidney suggests that the bile is a route of elimination in the pig. Brand (2011) reported that poultry also use bile as a route of elimination.

Brand (2011) reported much higher levels of MEL in the breast, kidney and bile of broilers and poults fed 1.00 % MEL than was observed in pigs receiving 1.0% MEL in the current study. These differences in MEL residue concentrations between poultry and swine can possibly be explained by species differences in how poultry and swine absorb and process MEL. Another possible explanation could be the size difference between a three wk old bird and eight wk old barrows. Assuming the same amount of MEL is absorbed, MEL would be more concentrated in the smaller (550 g) bird than in the larger (21,730 g) barrows. Differences in feed intake between an eight wk old pig and three wk old duck could also explain the difference in MEL reside levels. Other possible explanations include: 1) differences in how species absorb and transport MEL from their gastrointestinal tract in to their body; 2) differences in how MEL is filtered by the liver; 3) the degree to with hepatobiliary recirculation occurs; 4) differences in the kidneys ability to filter MEL from the blood; 5) and finally the ability of the kidneys to excrete MEL into the urine without precipitation occurring. Water flow through the kidney could play a large factor in the ability of the kidney to excreta MEL into the urine without precipitation occurring, thus decreasing renal damage. Therefore, animal/species with higher water consumption resulting in larger urine output might be better able to flush MEL out of their kidney preventing precipitation.

Performance of the MEL + CYA (0.75 % of each compound) fed pigs was much lower than the performance of pigs fed 0.75 % MEL. Relative kidney weights were

dramatically increased in MEL + CYA fed pigs (approximately double of controls). Blood parameters such as BUN and CREAT were elevated well above that of controls pigs (11 and 7 times higher, respectively), indicating renal failure. MEL + CYA pigs also had elevated WBC counts and WBC present in the urine. Histopathology of kidneys from the MEL + CYA pigs revealed several lesions and crystals that were golden brown to yellow in color. Crystals from the MEL + CYA pigs were visible under polarized light, a characteristic consistent with MEL + CYA crystals (Brand, 2011; Brown et al., 2007; Puschner et al., 2007; Reimschuessel et al., 2008) These data are in agreement with previous research in which a combination of MEL and CYA was shown to be more toxic to mammals than MEL alone (Dobson et al., 2008; Puschner et al., 2007; Reimschuessel et al., 2008; Stine et al., 2011). However, in poults, the addition of CYA to diets containing MEL appeared to reduce the negative effects of MEL on feed intake and body weight gain Brand (2011). Furthermore, the addition of CYA to diets containing MEL appears to ameliorate the negative effects of MEL on performance and mortality rates in young Pekin ducks (Chapter 4).

MEL residue levels in the kidney, muscle, and bile of MEL + CYA fed pigs were lower than tissues from 0.75 % MEL fed pigs. This suggests that while a combination of MEL and CYA is more toxic to the pig, there is less absorption of the MEL-CYA acid complex from the gastrointestinal tract as compared to MEL alone. This decrease in bioavailability of MEL-CYA complex as compared to MEL alone, due to precipitation of MEL-CYA in the gastrointestinal tract, was first suggested by Reimschuessel et al. (2008).

In conclusion dietary levels of ≤ 1.00 % MEL was toxic to young barrows fed contaminated diets for 21 days. However, MEL levels between 1.00 % and 1.25 % of the diet decreased growth performance of young barrows. There appeared to be no effect from the treatment on kidney function, as determined by serum levels of BUN and CREAT, and histopathology results. These results are in agreement with results from similar experiments conducted on mammals by Melnick et al. (1984), Reimschuessel et al. (2008), and Stine et al. (2011). Residue levels in the kidney and bile suggest that MEL may be eliminated via the bile as well as the kidney. The use of the hepatobiliary system as an alternative route of elimination has been suggested to occur in poultry by Brand (2011). More research is needed to determine if conversion of MEL to CYA can occur by microbes in the gastrointestinal tracts of mammals and/or by metabolism in mammals. Finally more research is needed to determine if the liver/gallbladder are affected by MEL consumption.

Table 5.1. Ingredients and nutrient composition of basal ration

Ingredient	Composition (%)
Corn	49.77
Soybean Meal	27.50
Whey, Dried	10.00
Plasma	2.50
Choice White Grease	5.00
Dicalcium Phosphate	2.05
Limestone	0.87
Vitamin Premix[1]	0.50
Salt	0.20
L-Lysine	0.15
Trace Mineral Premix[2]	0.15
DL-Methionine	0.065
Sand	1.25
Total	100.00
Nutrient composition (calculated)	
Crude Protein (%)	20.5
Metabolizable Energy (kcal/Kg)	1580
Lysine (SID, %)	1.20
Methionine (SID, %)	0.34
Methionine + Cysteine (SID, %)	0.68
Threonine (SID, %)	0.72
Calcium (%)	0.95
Phosphorus (% Av.)	0.55

[1]Vitamin premix provided (mg/kg of diet): retinyl acetate, 11,000 IU; cholecalciferol, 1,100 IU; DL-α-tocophereryl acetate, 44.1 IU; menadione Na dimethylpyrimidinol bisulfate, 4.0; vitamin B_{12}, 30.3 µg; riboflavin, 8.3 mg; D-Ca-pantothenate, 28.1 mg; nicotinamide, 33.1 mg; choline chloride, 551.3 mg; D-biotine, 0.22 mg; folic acid, 1.65 mg

[2]Trace mineral premix provided (mg/kg of diet): Zn, 165 mg (ZnSO4); Fe, 165 mg (FeSO4H2O); Cu, 16.5 mg (CuSO45H2o); Mn, 33 mg (MnSO4); I, 0.3 mg Ca(IO3)2; Se, 0.3 mg (Na2Se03).

SID = Standardized ileal digestible basis.

Table 5.2. Effects of melamine on performance and organ weights of young barrows

Treatment[1]		Response Variables[2]								
Melamine (%)		BWG (kg)	ADG (kg)	FI (kg)	G:F (kg:kg)	Mortality (%)	Liver wt. (g)	RLiver (%)	Kidney wt. (g)	RKidney (%)
Basal Diet + 0.00% MEL		12.76[a]	0.61[a]	20.68	0.63[ab]	0	614	2.64	129	0.56
Basal Diet + 0.25% MEL		11.90[a]	0.57[a]	18.23	0.65[a]	0	603	2.67	145	0.64
Basal Diet + 0.50% MEL		11.86[a]	0.56[a]	18.63	0.64[ab]	0	600	2.66	131	0.58
Basal Diet + 0.75% MEL		11.41[ab]	0.54[ab]	17.46	0.65[a]	0	576	2.63	128	0.59
Basal Diet + 1.00% MEL		9.50[b]	0.45[b]	17.48	0.56[bc]	0	592	2.97	121	0.60
Basal Diet + 1.25% MEL		9.44[b]	0.45[b]	18.07	0.53[c]	0	560	2.84	126	0.63
0.75%MEL + 0.75%CYA[3]		7.76	0.43	15.26	0.51	0	499	2.48	235	1.15
ANOVA:[4]	S.E.M.	0.75	0.04	1.69	0.03	-	37	0.14	10	0.04
	P-value	0.0205	0.0205	0.7769	0.0427	-	0.9191	0.4384	0.6305	0.5644
Regression:[5]	L: *P*-Value	0.0005	0.0005	0.2326	0.0076	-	0.2761	0.1078	0.2719	0.4130
	Q: *P*-Value	0.6076	0.6076	0.3619	0.0407	-	0.9365	0.6352	0.7778	0.9132

[1]Treatments were the addition of melamine, in percent indicated, to a basal diet. Experimental diets were fed for 21 day, except for MEL + CYA pig, which received diets for 18 days.
[2]Data are means of five replicate pens with one barrow per pen.
[3]Values from pigs fed MEL + CYA were not included in statistical analysis. Diet was inclusion of 0.75 % MEL and 0.75 % CYA added to the basal diet.
[4]One way analysis of variance values.
[5]Regression: Linear (L) or quadratic (Q) regression.
[a-c] Means within a column with no common superscript are different ($P < 0.05$).
BWG = body weight gain; ADG = average daily gain; FI = feed intake; G:F = gain to feed; RLiver = relative liver weight; RKidney = relative kidney weight; MEL = melamine; CYA = cyanuric acid.

Table 5.3. Effects of melamine on blood variables of young barrows

Treatment[1]		Response Variables[2]							
Melamine (%)		WBC (10^3/µL)	RBC (10^6/µL)	HGB (g/dL)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	PLT (10^3/µL)
Basal Diet + 0.00% MEL		22.46	6.96	12.28	41.58	59.80[a]	17.70	29.60	446
Basal Diet + 0.25% MEL		25.63	6.98	11.96	40.14	57.64[ab]	17.18	29.78	366
Basal Diet + 0.50% MEL		25.95	6.77	11.78	39.46	58.38[a]	17.46	29.88	354
Basal Diet + 0.75% MEL		28.95	7.29	11.88	39.38	54.08[b]	16.32	30.18	412
Basal Diet + 1.00% MEL		26.35	7.12	12.14	40.58	56.94[ab]	17.04	29.90	419
Basal Diet + 1.25% MEL		23.40	6.89	11.28	37.58	54.56[b]	16.38	29.98	439
0.75%MEL + 0.75%CYA[3]		37.47	6.89	10.75	32.65	47.15	15.55	33.00	580
ANOVA:[4]	S.E.M.	3.36	0.28	0.47	1.63	1.27	0.45	0.34	44
	P-value	0.7945	0.8188	0.7464	0.6358	0.0295	0.2085	0.8880	0.5950
Regression:[5]	L: *P*-Value	0.7153	0.7966	0.2675	0.1675	0.0068	0.0393	0.3475	0.6123
	Q: *P*-Value	0.1590	0.6577	0.8254	0.9847	0.5499	0.7992	0.4969	0.1536

[1]Treatmensts were the addition of melamine, in percent indicated, to a basal diet. Experimental diets were fed for 21 day, except for MEL + CYA pig, which received diets for 18 days.
[2]Data are means of five replicate pens with one barrow per pen.
[3]Values from pigs fed MEL + CYA were not included in statistical analysis. Diet was inclusion of 0.75 % MEL and 0.75 % CYA added to the basal diet.
[4]One way analysis of variance values.
[5]Regression: Linear (L) or quadratic (Q) regression.
[a-b] Means within a column with no common superscript are different ($P < 0.05$).
WBC = white blood cell count; RBC = red blood cell count; HGB = hemoglobin; HCT = hematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; PLT = platelet count; CBC = complete blood count; MEL = melamine; CYA = cyanuric acid.

Table 5.4. Effects of melamine on serum proteins, bilirubin, and select enzymes of young barrows

	Treatment[1]							ANOVA:[4]		Regression:[5]	
	Melamine (%)										
Response Variables[2]	0.00% MEL	0.25% MEL	0.50% MEL	0.75% MEL	1.00% MEL	1.25% MEL	MEL + CYA[3]	S.E.M	*P*-Value	Linear *P*-Value	Quadratic *P*-Value
GLU	134	136	139	127	122	117	166	6	0.1436	0.0124	0.2182
AST	24.2	29.8	38.6	36.8	37.8	49.2	26.0	5.9	0.1033	0.0049	0.9670
BUN	10.2	8.6	10.2	9.8	10.0	8.8	111	1.3	0.8944	0.7568	0.7504
CREAT	0.84	0.76	0.82	0.88	0.86	0.78	5.8	0.05	0.6179	0.8971	0.5304
ALB	3.30	3.18	3.26	3.14	3.06	2.96	3.60	0.14	0.5698	0.0651	0.6671
GLOB	2.02	1.98	2.08	2.22	2.20	2.30	2.75	0.19	0.8166	0.1549	0.8947
T BIL	0.10	0.10	0.12	0.30	0.22	0.20	0.15	0.08	0.4496	0.1324	0.5017
D BILI	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.008	0.4389	0.3973	0.7955
TP	5.32	5.16	5.34	5.36	5.26	5.26	6.35	0.16	0.9494	0.9874	0.8170
GGT	33.0	31.6	27.4	30.4	27.8	33.6	30.5	2.6	0.4475	0.8030	0.0821
CPK	1014	1567	2262	914	746	1345	627.5	368	0.0794	0.5252	0.4010

[1]Treatments were the addition of melamine, in percent indicated, to a basal diet. Experimental diets were fed for 21 day, except for MEL + CYA pig, which received diets for 18 days.
[2]Data are means of five replicate pens with one barrow per pen.
[3]Values from pigs fed MEL + CYA were not included in statistical analysis. Diet was inclusion of 0.75 % MEL and 0.75 % CYA added to the basal diet.
[4]One way analysis of variance values.
[5]Regression: Linear (L) or quadratic (Q) regression.
GLU = glucose (mg/dL); AST = aspartate aminotransferase (U/L); BUN = blood urea nitrogen (mg/dL); CREAT = creatinine (mg/dL); TP = total protein (g/dL); GLOB = globulin; T BIL = total bilirubin (mg/dL); D BILI = direct bilirubin (mg/dL); ALB = albumin (g/dL); GGT = gamma-glutamyl transpeptidase (U/L); CPK = creatine phosphokinase (U/L); MEL = melamine; CYA = cyanuric acid.

Table 5.5. Effects of melamine on serum mineral concentrations of young barrows

	Treatment[1]							ANOVA:[4]		Regression:[5]	
	Melamine (%)										
Response Variables[2]	0.00% MEL	0.25% MEL	0.50% MEL	0.75% MEL	1.00% MEL	1.25% MEL	MEL + CYA[3]	S.E.M	*P*-Value	Linear *P*-Value	Quadratic *P*-Value
Ca	11.6	11.0	11.2	11.0	10.3	10.6	11.5	0.3	0.0542	0.0053	0.8687
P	11.2	10.8	10.7	10.6	10.3	11.2	12.8	0.5	0.7652	0.6582	0.2056
Mg	2.52	2.44	2.46	2.42	2.36	2.38	3.30	0.15	0.9762	0.4036	0.8879
Na	148	144	142	147	144	141	142	3	0.6225	0.2379	0.9891
K	6.24	5.82	5.92	5.82	6.00	6.86	6.20	0.54	0.7361	0.4137	0.1617
Cl	104	103	102	105	102	101	96	2	0.8319	0.4993	0.7971

[1]Treatments were the addition of melamine, in percent indicated, to a basal diet. Experimental diets were fed for 21 day, except for MEL + CYA pig, which received diets for 18 days.
[2]Data are means of five replicate pens with one barrow per pen.
[3]Values from pigs fed MEL + CYA were not included in statistical analysis. Diet was inclusion of 0.75 % MEL and 0.75 % CYA added to the basal diet.
[4]One way analysis of variance values.
[4]Regression: Linear (L) or quadratic (Q) regression.
Ca = calcium (mg/dL); P = phosphorus (mg/dL); Mg = magnesium (mg/dL); Na = Sodium (mEq/L); K = potassium (mEq/L); Cl = chloride (mEq/L); M = melamine; CYA = cyanuric acid.

Table 5.6. Melamine concentrations in the kidney, muscle, and bile of young barrows

Treatment[1]		Response Variables[2]		
Melamine (%)		Kidney[2] (ppm)	Muscle - hind quarter[3] (ppm)	Bile[4] (ppm)
Basal Diet + 0.00% MEL		ND[d]	ND[c]	ND[c]
Basal Diet + 0.25% MEL		41[c]	12[c]	20[c]
Basal Diet + 0.50% MEL		75[b]	33[b]	63[b]
Basal Diet + 0.75% MEL		75[b]	33[b]	77[b]
Basal Diet + 1.00% MEL		143[a]	62[a]	85[b]
Basal Diet + 1.25% MEL		125[a]	57[a]	126[a]
0.75%MEL + 0.75%CYA[5]		32	17	46
ANOVA:[6]	S.E.M.	0.06	2	9
	P-value	< 0.0001	< 0.0001	< 0.0001
Regression:[7]	L: *P*-Value	< 0.0001	-	< 0.0001
	Q: *P*-Value	< 0.0001	-	0.6718

[1]Treatments were the addition of melamine, in percent indicated, to a basal diet. Experimental diets were fed for 21 day, except for MEL + CYA pig, which received diets for 18 days.
[2] Data are means of four replicate pens with one barrow per pen. Statistical analysis was performed on transformed data (log10).
[3] Data are means of four replicate pens with one barrow per pen. Statistical analysis was performed on transformed data (ranked data).
[4]Data are means of five replicate pens with one barrow per pen.
[5]Values from pigs fed MEL + CYA were not included in statistical analysis. Diet was inclusion of 0.75 % MEL and 0.75 % CYA added to the basal diet.
[6]One way analysis of variance values.
[7]Regression: Linear (L) or quadratic (Q) regression.
[a-d] Means within a column with no common superscript are different ($P < 0.05$).
MEL = melamine; CYA = cyanuric acid; ND = none detected.

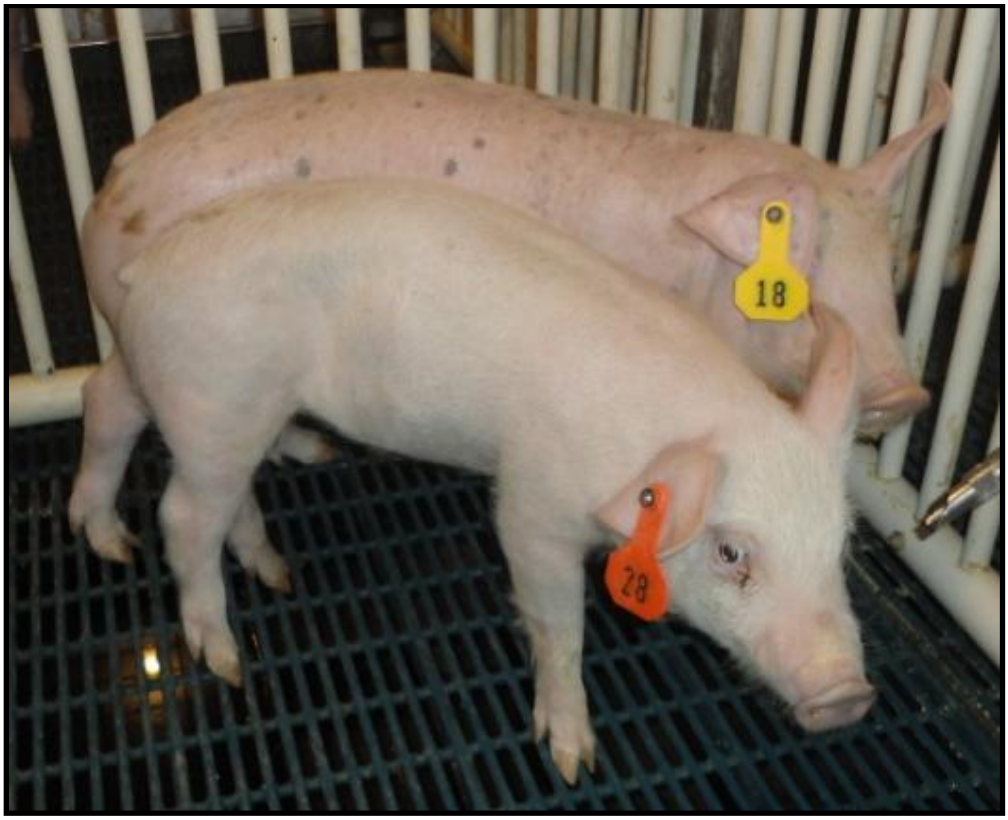


Figure 5.1. Effects of graded levels of melamine on barrow performance. Back pig (tag 18) was fed a basal diet without MEL (control pig) for 21 days. Front pig (tag 28) received the basal diet plus 1.25 % MEL for 21 days.

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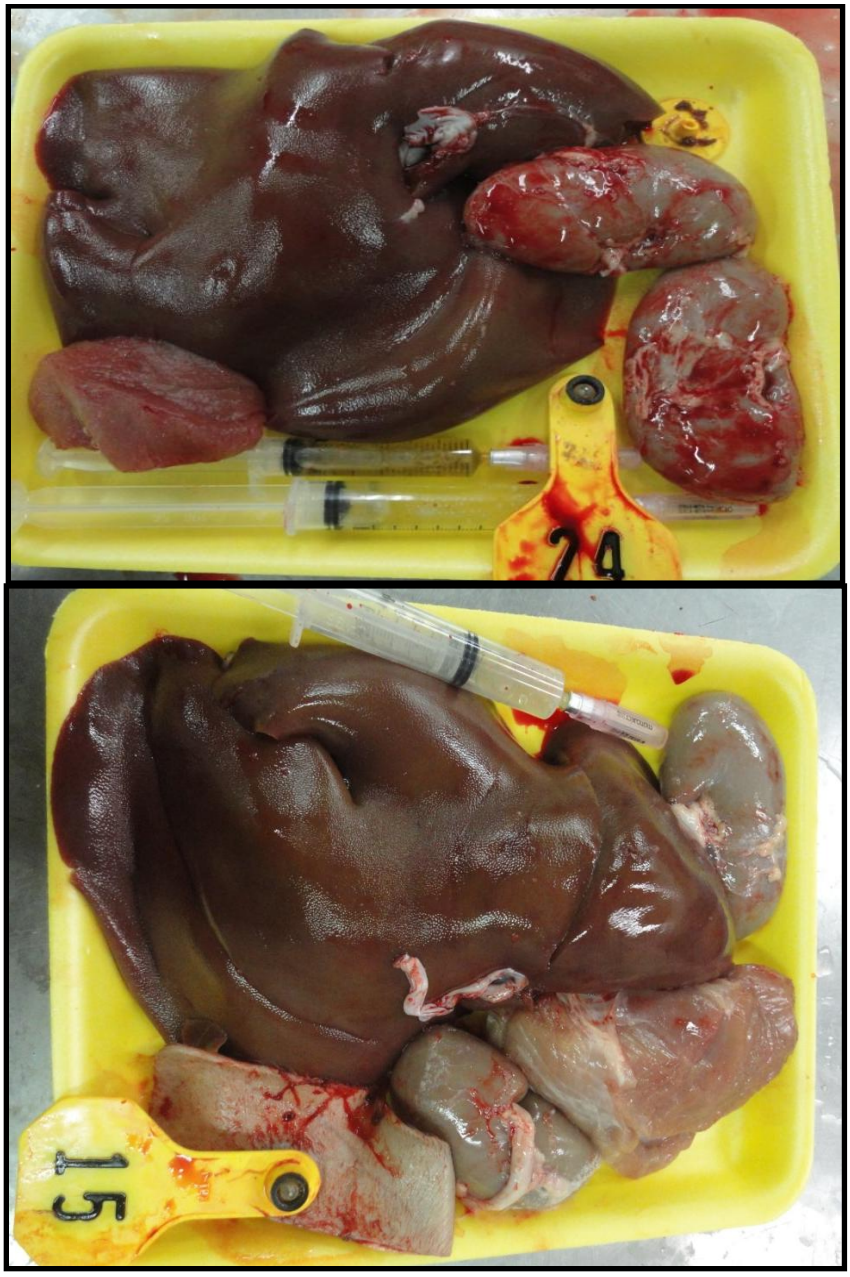

Figure 5.2. Effects of melamine on the gross appearance of organs. Top photo (tag 24) shows the liver and kidneys from a pig fed a basal diet without MEL (control pig). Bottom photo (tag 15) shows the liver and kidney from a pig fed the basal diet plus 1.25 % MEL.

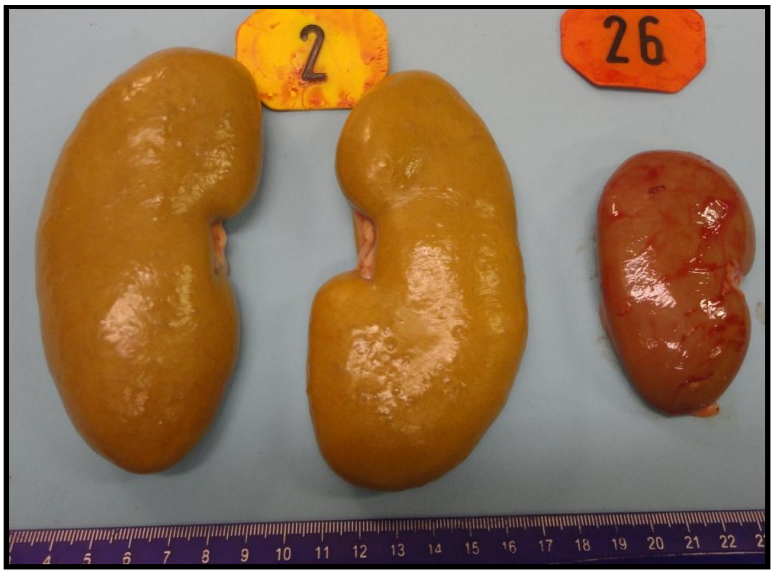

Figure 5.3. Effects of melamine and melamine plus cyanuric acid on the kidneys of barrows. Kidneys on left (tag 2) are from a pig that received a diet that contained 0.75 % MEL and 0.75 % CYA. The kidney on the right (tag 26) is from a pig that received the basal diet without MEL (control pig).

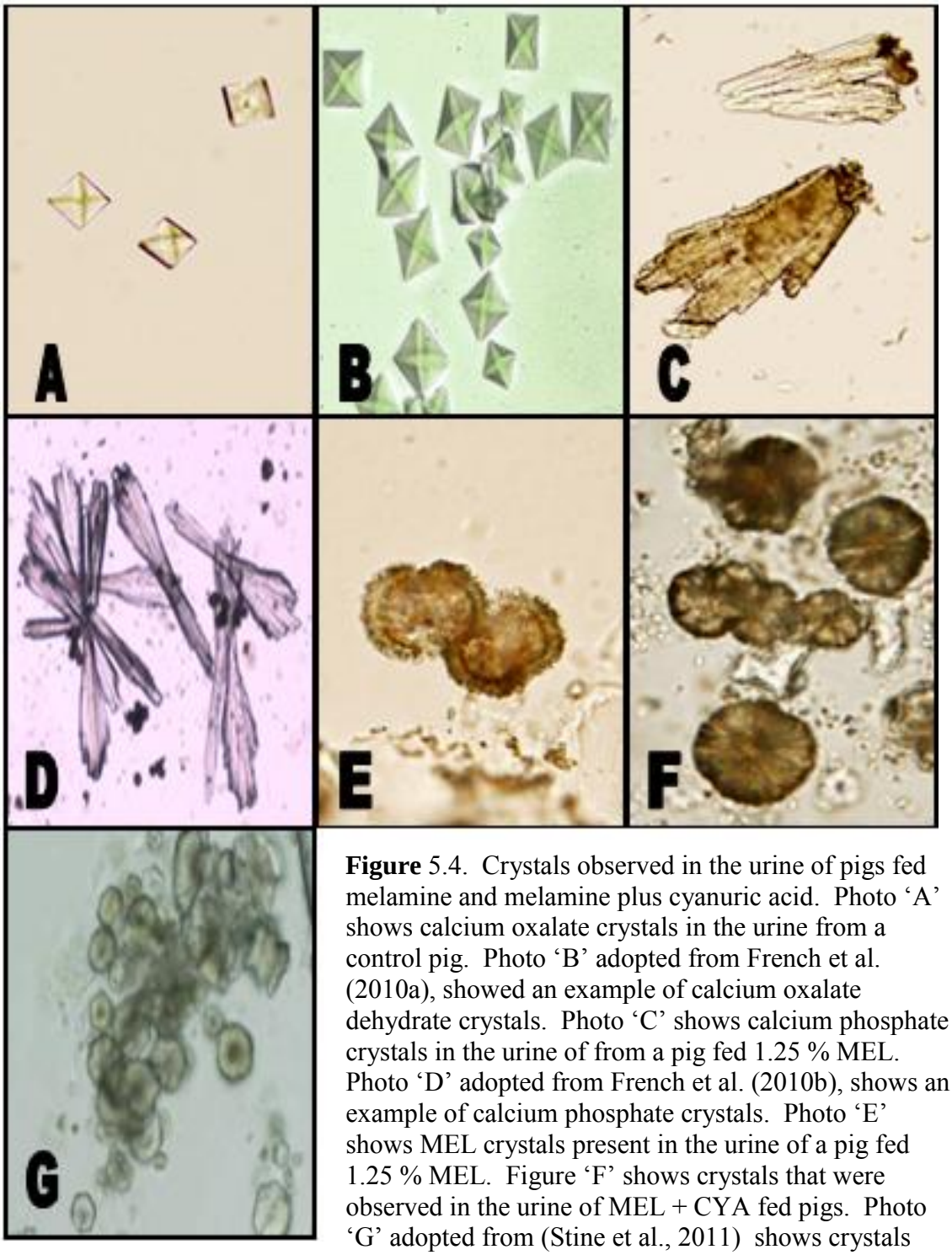

Figure 5.4. Crystals observed in the urine of pigs fed melamine and melamine plus cyanuric acid. Photo 'A' shows calcium oxalate crystals in the urine from a control pig. Photo 'B' adopted from French et al. (2010a), showed an example of calcium oxalate dehydrate crystals. Photo 'C' shows calcium phosphate crystals in the urine of from a pig fed 1.25 % MEL. Photo 'D' adopted from French et al. (2010b), shows an example of calcium phosphate crystals. Photo 'E' shows MEL crystals present in the urine of a pig fed 1.25 % MEL. Figure 'F' shows crystals that were observed in the urine of MEL + CYA fed pigs. Photo 'G' adopted from (Stine et al., 2011) shows crystals observed in a pig fed MEL + CYA.

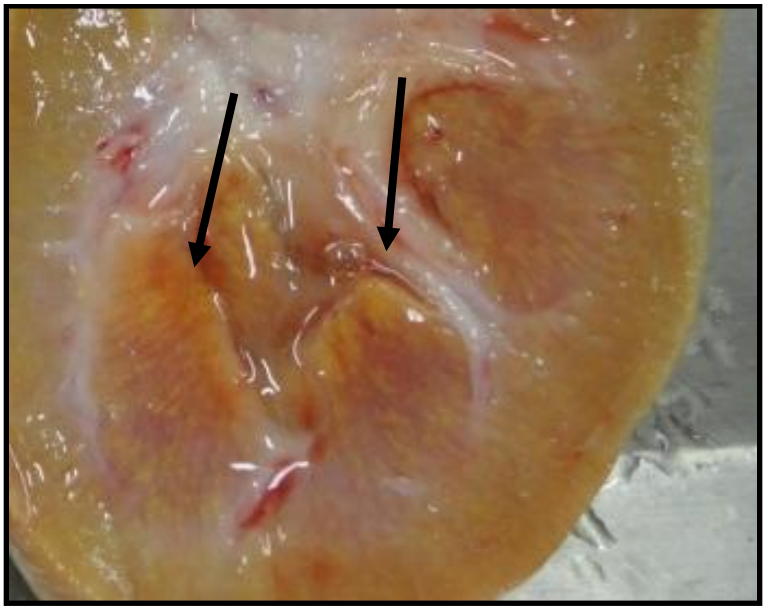

Figure 5.5. Effects of melamine plus cyanuric acid on the kidneys of young barrows. Cross section shows the effects of MEL + CYA (0.75 % of each compound) for 18 days on the kidneys of young barrows. Yellowish to golden discoloration, present at arrows, is due to the presence of crystal deposits.

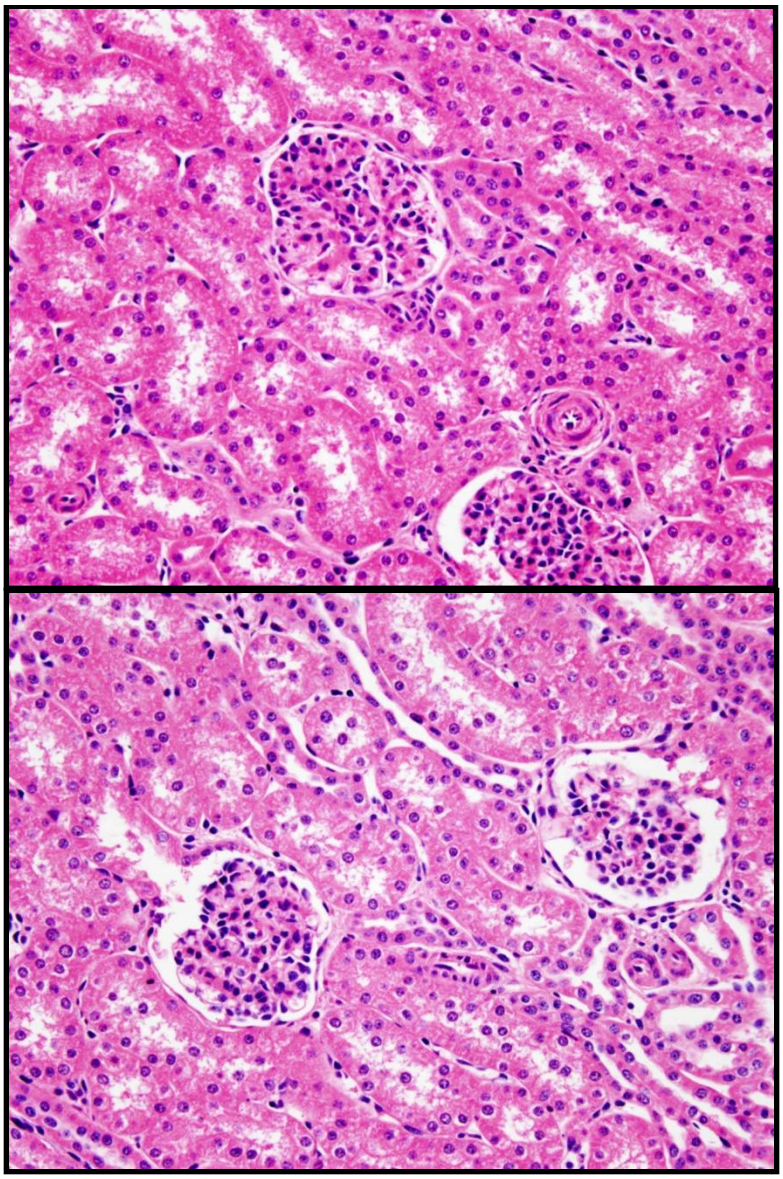

Figure 5.6. Microscropic effects of melamine in the kidney of barrows. No changes were noted at the cellular level between treatments. Top photo (A) is from a pig fed basal diet without MEL. Bottom photo (B) is from a pig fed basal diet plus 1.25 % MEL.

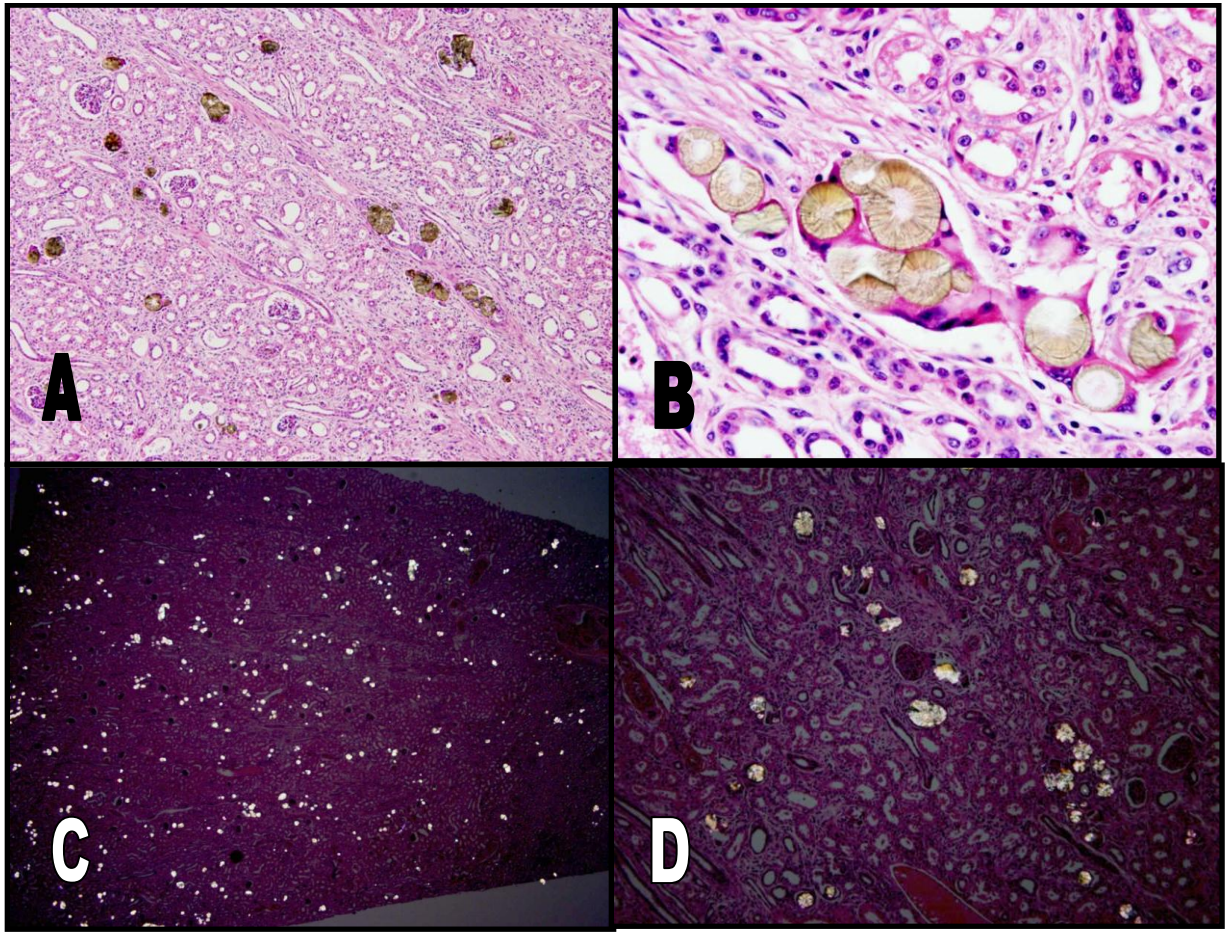

Figure. 5.7. Effects of melamine plus cyanuric acid on the kidneys of young barrows. Photo 'A' and 'B' show golden-brown radial spheroid crystals in the kidneys of combination fed pigs. These crystals were visible under polarized light (photos 'C' and 'D').

Chapter 6

Conclusion

Two experiments were conducted to determine the individual and combined effects of melamine (MEL) and cyanuric acid (CYA) in young Pekin ducks. A third experiment was conducted to determine the effects of MEL in young weanling pigs (barrows). All experiments were conducted at the University of Missouri – Columbia. The animal care and use protocol was reviewed and approved by the University of Missouri Animal Care and Use Committee before each experiment was performed.

Experiment one determined the effects of graded levels of MEL (0.00, 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, and 2.25 % of the diet) in Pekin ducks from hatch to 21 days of age. Compared to controls, mortality was higher in ducks fed ≥ 2.00 % MEL. Feed intake and body weight gain was lower than controls in ducks fed ≥ 1.00 % MEL. Compared to controls, relative kidney weights were heavier in ducks fed ≥ 1.00 % MEL. All blood parameters measured (glucose, albumin, total protein, globulin, aspirate transaminase, gamma glutamyltranserase, and uric acid) increased in a linear fashion as dietary MEL inclusion increased. MEL residue levels were highest in the bile, followed by the kidney, and finally the muscle. The above data, along with the mild to severe MEL induced lesions observed in the kidney of broilers fed ≥ 1.00 % MEL show that renal failure induced by MEL toxicity can cause a decrease in performance (≥ 1.00 % of the diet) of young Pekin ducks, and eventually death (≥ 2.00 %). Lesions included dilation of the embryonal nephrons with eosinophilic to basophilic casts present. Some of the casts contained spherical crystals. These data also confirm that young Pekin ducks

use bile as a route for eliminating MEL from their bodies. Finally, residue levels in the breast muscle are not high enough to pose a health risk to humans, even when such high dietary concentrations are consumed.

Experiment two studied the effects of MEL and CYA, alone or in combinations, on Pekin ducks from hatch to 21 days of age. Diets included: 1) Control, no MEL or CYA (basal diet); 2) basal diet + 0.50% MEL; 3) basal diet + 1.00 % MEL; 4) basal diet + 1.50 % MEL; 5) basal diet + 0.50 % CYA; 6) basal diet + 1.00 % CYA; 7) basal diet + 1.50 % CYA; 8) basal diet + 0.50 % MEL and 0.50 % CYA; 9) basal diet + 1.00 % MEL and 1.00 % CYA; 10) basal diet + 1.50 % MEL and 1.50 % CYA. Only treatments fed MEL alone caused mortality. Ducks fed ≥ 1.00 % MEL had lower feed intake and weight gain, increased relative kidney weights, and increased serum ALB, TP, GLOB, and UA levels as compared to the controls. The inclusion of up to 1.50 % CYA or MEL + CYA (up to 1.50 % of each) did not cause any of the previously mentioned variables to be different from controls. Histopathology revealed kidneys from ≥ 1.00 % MEL fed ducks to be have lesions compatible with MEL toxicity, while the kidneys from ducks fed up to 1.50 % CYA and combinations of MEL + CYA (≤ 1.50 % of each compound) were unaffected by MEL inclusion. MEL associated lesions included dilation of the embryonal nephrons and collecting tubules. Eosinophilic to basophilic casts, some containing spherical eosinophilic crystals, were present in some of the embryonal nephrons and collecting tubules. These results suggest that: 1) CYA up to 1.50 % of a duck's diet is not toxic to young Pekin ducks; 2) inclusion of CYA to a diet that contains MEL (a one-to-one ratio of MEL to CYA) can alleviate the negative effects of MEL on young Pekin ducks. Finally, from the residue values collected on the breast muscle,

kidney, and bile, it appears that: 1) The bile can be used to eliminate MEL from the body; 2) the ability of CYA to alleviate the effects of MEL in young ducks appears to be caused by the precipitation of a MEL-CYA complex in the gastrointestinal tract, thus reducing the bioavailability of MEL.

Experiment three evaluated the effects of feeding MEL to weanling barrows for 21 days. MEL was included in the diet at 0.00, 0.25, 0.50, 0.75, 1.00, and 1.25 %. Two additional pigs were fed diets that contained a combination of 0.75 % MEL + 0.75 % CYA. No mortality occurred during the 21 day test period. Compared to controls, gain and gain to feed were lower in treatments fed ≥ 1.00 % and 1.25 % MEL, respectively. Blood urea nitrogen, creatinine levels and relative kidney weight were not affected by the dietary inclusion of up to 1.25 % MEL. The average daily gain of the pigs fed the MEL + CYA combination was 1.4 fold lower than the average daily gain of controls. MEL + CYA pigs had blood urea nitrogen and creatinine levels that were 11 and 7 fold higher than levels in control pigs. MEL + CYA pigs also had heavier relative kidney weights than controls. Crystals found in the kidneys of MEL + CYA pigs were round, golden brown in color, and visible under polarized light. The only clinical significant difference noted in the urine samples among treatments fed MEL was the presence of crystals in one pig fed 1.25 % MEL. The crystals found in the urine of the pig fed 1.25 % MEL was similar in appearance to those found in the urine of pigs fed MEL + CYA. Residue levels in the kidney, muscle, and bile were lower in pigs fed MEL + CYA (0.75 % MEL + 0.75 % CYA) than pigs fed ≥ 0.50 % MEL.

Data collected on pigs fed up to 1.25 % MEL suggest that MEL levels between 1.00 and 1.25 % of a weanling pigs diet decreased growth performance but did not cause

mortality. Up to 1.25 % MEL does not appear to affect kidney size or function. In contrast, the combination of CYA + MEL causes renal damage, indicated by increased kidney size and elevated blood urea nitrogen and creatinine. Crystals found in the urine of one pig fed 1.25 % MEL was similar in structure to crystals found in MEL + CYA fed pigs. This suggests that conversion of MEL to CYA may occur via microorganisms in the gastrointestinal tract or via metabolism. Residue values suggest that swine may also eliminate MEL via the bile. It appears that the inclusion of CYA in a diet that contains MEL is more toxic to pigs than MEL alone. However, due to precipitation of the MEL-CYA complex in the gastrointestinal tract it appears that these pigs absorbed less MEL than MEL only fed pigs. Finally, muscle residue values show that concentrations of MEL in the muscle of swine from the current study would result in a daily intake of MEL that is below the TDI of 0.063 mg/kg BW/day set by the FDA.

The results of these studies document the individual and combined effects of MEL and CYA on young Pekin ducks and weanling barrows fed dietary treatments for 21 days. In young Pekin ducks ≥ 1.00 % MEL can cause a decrease in performance and changes in serum chemistry values indicating decreased renal function. Up to 1.50 % CYA does not affect performance of young Pekin ducks and the addition of CYA to a diet that contains MEL can alleviate the negative effects of MEL. Lesions detected in ducks fed ≥ 1.00 % MEL are similar to lesions documented in broilers, poults, and cats, such as pale and enlarged kidneys with crystals present in the lumina of the collecting ducts/tubules. In young barrows, up to 1.25 % MEL did not cause changes in renal appearance, changes in blood urea nitrogen or creatinine levels, nor did it cause mortality during the treatment period. However, levels ≥ 1.00 % MEL did cause a reduction in

body weight gain over the 21 day experimental period. At the same time the combination of 0.75 % MEL + 0.75 % CYA caused barrows to have lower body weight gains than controls and higher blood urea nitrogen and creatinine levels than controls.

From the data collected it appears that both young Pekin ducks and barrows use bile as a route to eliminate MEL from their body. Feeding CYA in combination with MEL causes precipitation of a MEL-CYA complex in the gastrointestinal tract of the animals, thus decreasing the concentration of MEL in the muscles, kidneys, and bile as compared to animals that only consume MEL. It should be noted that feeding CYA in combination with MEL reduces the negative effects of MEL on renal function in young Pekin ducks, but is more detrimental to renal function in young barrows.

Finally, more research is needed to determine why in some species the combination of CYA added to a diet contaminated with MEL can prevent the negative effects of MEL (poults and ducks) but in other species the combinations of MEL + CYA is more toxic than MEL alone (pigs, cats, dogs) and why MEL accumulates more in some species than others. Possible explanations, and areas for additional research, include: 1) differences in how species absorb and transport MEL from their gastrointestinal tract in to their body; 2) differences in how MEL is filtered by the liver; 3) the degree to with hepatobiliary recirculation occurs; 4) differences in the kidney ability to filter MEL from the blood; 5) and finally the ability of the kidneys to excrete MEL into the urine without precipitation occurring. Water flow through the kidney could play a large factor in the ability of the kidney to excreta MEL into the urine without precipitation occurring, thus decreasing renal damage. Therefore, animal/species with higher water consumption

resulting in larger urine output might be better able to flush MEL out of their kidney preventing precipitation.

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