EFFECTS OF DIETARY MELAMINE AND CYANURIC ACID IN YOUNG BROILERS AND TURKEY POULTS

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by

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

EFFECTS OF DIETARY MELAMINE AND CYANURIC ACID
IN YOUNG BROILERS AND TURKEY POULTS

Presented by Lindsay Marie Brand

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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DEDICATION

This thesis is dedicated to my parents, Keith and Lola Brand. Their confidence, patience, love, and support have driven me to become a well-educated, moral, ethical, and determined individual. Their determination and success have influenced me in every aspect of my life, and without their guidance I would have never made it to this point.

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ABSTRACT

Six studies were conducted to determine the individual and combined toxicity of melamine and cyanuric acid (up to 3.0%) in young broilers and turkey poults. Melamine alone caused reduced growth, increased kidney weights, and renal lesions in broilers and poults fed 1.0% or higher melamine. Renal histopathology revealed nonpolarizable melamine crystals in the collecting tubules and ducts, which contributed to renal failure. Residual melamine concentrations were highest in kidney, followed by liver, bile, and breast muscle. No toxicity was observed in broilers or turkeys fed up to 3.0% cyanuric acid alone. Growth was reduced in broilers fed combinations of melamine and cyanuric acid up to 3.0%, however no mortality was observed. In turkeys, the addition of cyanuric acid to diets containing melamine ameliorated the toxic effects observed when poults were fed melamine alone. Broilers and turkeys fed combinations of melamine and cyanuric acid had renal crystals viewable under normal and polarized light.

CHAPTER 1

Introduction

Wheat gluten products incorporated in animal feedstuffs in 2004 and 2007 and in infant formulas in 2008 were reported to have been intentionally contaminated with melamine (1,3,5-triazine-2,4,6-triamine) and its related triazine, cyanuric acid (1,3,5-triazine-2,4,6-trihydroxy) in order to artificially increase nitrogen content (Brown et al., 2007; Burns, 2007a; FDA 2007b; Food Report, 2008; Ingelfinger, 2008; OSHA, 2010). Toxic effects were quickly detected in many species (particularly cats, dogs and humans) consuming products that were later found to be contaminated with melamine (NIOSH, 2006; OSHA, 2009). Companion animals fed wheat gluten products contaminated with melamine were diagnosed with renal failure, exacerbated by crystal formation in the renal tubules, which lead to high mortality rates among affected cats and dogs (Brown et al., 2007; Puschner et al., 2007). Upon further investigation, it was found that poultry and swine had also consumed melamine contaminated feed in the form of pet food byproducts, although melamine concentrations were considered too low to cause health problems (Burns, 2007a; FDA, 2007a; Filigenzi et al., 2007).

The main objective of the research presented in this thesis was to determine if melamine or cyanuric acid alone, or in combinations up to 3%, would cause toxic effects in young broilers and turkeys. Additionally, if toxicity was noted, what parameters

would be negatively affected and how comparable would the results be to those that were recorded in animals such as cats and dogs. Secondly, residue levels of melamine in liver, kidney, breast muscle, and bile were determined to better understand the kinetics of the oral administration of melamine and/or cyanuric acid.

CHAPTER 2

Literature Review

Chemistry and Use

Melamine (1,3,5-triazine-2,4,6-triamine) is a white, crystalline compound that can be purchased in powder form (NIOSH, 2006; OSHA, 2009). The molecular formula for melamine is $C_3H_6N_6$ (NIOSH, 2006; OSHA, 2009; Safety Data, 2008; Figure 2.1). Millions of kilograms of melamine are produced in the Western world, including the United States (Ingelfinger, 2008), although most is produced in China (Turley, 2009). Melamine is nonflammable (NIOSH, 2006), incompatible with strong acids or oxidizing agents (Safety Data, 2008), and prior to 2007 was labeled as a nontoxic to minimally toxic compound (Tyan et al., 2009).

According to the Centers for Disease Control and Prevention (CDC, 2008), melamine is typically used as a synthetic chemical in industry for production of resins, foams, cleaning products, fertilizer (prohibited from use in the United States as a fertilizer), pesticides and fire retardant materials (FDA, 2007a; Lowy and Pritchard, 2008). Some inexpensive, plastic based dinnerware contains melamine, which when heated in the microwave may promote leaching of melamine into food, especially when acid is present, but amounts are considered insignificant (Buka et al., 2009; Dobson et al, 2008; Ingelfinger, 2008).

Because melamine's mass consists of 66% nitrogen, use of the Kjeldahl method or the Dumas method would suggest that melamine would be rich in protein (Ingelfinger, 2008). Kjeldahl and Dumas methods are performed by extracting nitrogen in a sample, the resulting nitrogen content is measured, and that quantity is multiplied by 6.25 to determine protein content (Mermelstein, 2009). These methods do not quantify or qualify the availability or nutritious aspect of the food product, hence, in the case of melamine can overestimate the amount of protein in the nitrogen rich yet nutritionally inadequate compound.

Cyanuric acid is also a triazine (1,3,5-triazine-2,4,6-trihydroxy; Figure 2.2) and has a molecular formula of $C_3H_3N_3O_3$ (OSHA, 2010). Deamination of melamine creates the compound ammeline, wherein the NH_2 is replaced with OH (Jutzi et al., 1982). Further deamination of ammeline results in the compound ammelide (Jutzi et al., 1982). When all NH_2 from the original melamine compound have been replaced with OH the resulting compound is cyanuric acid (Figure 2.3; Jutzi et al., 1982). Cyanuric acid is used in swimming pools as a stabilizer and protects chlorine from being degraded by the sun's ultraviolet rays (Cantú et al., 2001). Stabilizers, also known as conditioners, contain miniscule amounts of cyanuric acid but if a pool reaches more than 30 to 80 ppm it is recommended that the water be diluted with more water in order to reduce the cyanuric acid content ("Pool Manual").

Analysis

In an article for Foodtechnology, Mermelstein (2009) outlines various analyses for melamine and cyanuric acid detection. Liquid chromatography-mass spectrometry (LC-MS) is a widely available and reliable analysis for identifying melamine and cyanuric acid and has been a method recommended by the FDA (2007b). Brown et al. (2007) used LC-MS for detection of the triazine compounds in samples that had previously been collected and embedded in paraffin. Prior to running LC-MS on the samples, Brown et al. (2007) utilized an acetonitrile/water/diethylamine mixture in order to dissolve melamine cyanurate into individual melamine and cyanuric acid compounds. Dobson et al. (2008) and Ranganathan et al. (1999), upon histological examination of animal tissue sections, determined that the melamine-cyanuric acid combination was held in a multiple hydrogen bonded hexamer complex. The rosette complex was practically insoluble in water and in order to measure the quantity of the individual triazines, had to be disrupted with treatment by acid and heat.

He et al. (2008) developed a surface enhanced Raman spectroscopy (SERS) method using gold nanosubstrates to better detect melamine. The limit of detection (LOD) for this method was calculated to equal ~33 ppb in aqueous solution using the calculation of the highest melamine peak on the SERS spectra at 676 cm-1 (He et al., 2008). Lin et al. (2008) followed up the aforementioned study by testing high-performance liquid chromatography (HPLC) and Surface Enhanced Raman Spectroscopy (SERS) methods. The lowest LOD of melamine using HPLC was 0.1% in wheat gluten, 0.05% in chicken feed and cakes, and 0.07% in noodles, and recovery rates of melamine ranged from 81.8% to 111.2%. Lin et al. (2008) also tested and found

that raw and processed foods did not have different recovery rates. While HPLC is good for detecting melamine, the process unfortunately takes about 3 hours to complete, requires much cleanup and extraction, and is fairly expensive. Lin et al. (2008) found that SERS had a LOD of 0.033 μg/mL when melamine is measured in solution in comparison to the LOD of HPLC equal to 1 μg/mL. Lin et al. (2008) proposed running SERS to find samples testing positive for melamine and then using HPLC to further quantify those samples that test positive for melamine.

Another method of analysis that has been utilized by the FDA is that of gas chromatography/mass spectrometry (GS-MS). The GS-MS method of analysis is efficient in detecting melamine and cyanuric acid, and was employed by Cianciolo et al. (2008) in analysis of cat urine and kidneys. Due to strong ultraviolet absorbance, melamine can also be detected using a UV system such as an enzyme-linked immunosorbent assay (ELISA; Garber, 2008). Garber (2008) conducted a comparison among commercial ELISAs and found that the melamine specific ELISA had an LOD value of < 20 ng/mL. Companies are also becoming more interested in improving melamine detection and products such as Sprint^{TM} Rapid Protein Analyzer, developed by the CEM Corp., are concentrated on providing a rapid and production floor based quality control analyzer (Mermelstein, 2009).

Various properties of melamine have made it difficult to detect the compound and its triazine analogs. Melamine has a strong ultraviolet absorbance which allows for successful UV detection under acidic conditions, but UV detection and acidic conditions are complicated when detection involves complete and complex food compositions

(Garber, 2008). Another study using SERS discovered that the hydrogen bonds that form in milk products result in the formation of a compound that cannot easily be broken down via sonication, making it more difficult to analyze melamine concentrations (Liu et al., 2010a). Liu et al. (2010a) researched the keto and enol forms of cyanuric acid, recognizing that the different tautomers are measured at different spectrums and a complete analysis for cyanuric acid in milk has not yet been developed. Furthermore, Brown et al. (2007) discovered melamine crystals are subject to dissociation when stored in formalin for long periods of time and preservation of samples older than 6 months are not useful in melamine detection.

Recent Issues

Chinese exports have increased in the last 20 years by a factor of six: $4.5 billion in 1986 to $25.7 billion in 2006 (Mertens, 2009). Due to mass production and exportation of Chinese products it has become necessary for quality control to step up and ensure the production of safe products, but the rapid growth of production makes quality control difficult (Mertens, 2009; Turley, 2009).

On March 15, 2007, the adulteration of a vegetable protein was called to the attention of the FDA. The following day, March 16th, Menu Foods placed a voluntary recall on products that were imported from the Chinese food company, ChemNutra (Burns, 2007a; FDA 2007b). Seventeen days later, after many reports of animals falling ill and dying, ChemNutra eventually recalled their food products (Burns, 2007a; 2007b) and begun investigation of the supplier, Xuzhou Anying Biologic Technology (FDA,

2007b). Contaminated pet food, animal and fish feed, and vegetable proteins were recalled and instructed to be destroyed (FDA, 2007b). Two ingredients, wheat gluten and rice protein, were identified as the adulterated vegetable proteins and importation into the U.S. was halted until China could prove these products were no longer contaminated (Mermelstein, 2008).

Within the first four weeks of the recall, the FDA received more than 14,000 reports regarding the contamination scare (FDA, 2007b). From April 5 to June 6, 2007, 235 cats and 112 dogs (of which deaths were reported for 61% and 74%, respectively) were diagnosed with "pet food-induced nephrotoxicity" based on high concentrations of blood urea nitrogen (BUN) and creatinine (Burns, 2007b). The CEO of ChemNutra, Stephen Miller, stated that melamine was added to gluten purposefully and the person at fault may have assumed that there was less chance of being discovered if the adulterating was done to pet foods (Burns, 2007a). A Canadian company, Tembec, was implicated in 2007 for selling melamine contaminated binding agents used in fish and shrimp feed to aid in pelleting to a plant in Ohio (Martin, 2007). The products were exported, for the most part, but once Tembec realized some binding agents were sold nationally, they stopped the use of the melamine contaminated products (Martin, 2007).

Pigs and chickens that consumed contaminated pet food byproducts were traced to California, Illinois, Kansas, New York, North Carolina, and Utah (Burns, 2007a; FDA, 2007a). The decision made on April 28, 2007 to not recall meat of approximately 56,000 hogs and 80,000 chickens fed contaminated pet food waste was supported by the FDA

(2007a) due to the animals showing no signs of illness and no melamine was detected in the feed samples (Filigenzi et al., 2007). Research by Filigenzi et al. (2007) determined that melamine residues in the tissue of the affected animals were not high enough to pose a threat to human health.

Brown et al., (2007) found that animal tissues in the 2004 toxicity outbreak, which had originally been regarded as mycotoxicosis, exhibited identical histological, clinical and toxicological effects as those involved in the 2007 melamine toxicity outbreak. Animals associated with the 2004 and 2007 pet food recalls had lesions secondary to renal failure such as oral ulcers along the tongue and mineralization in the gastric mucosa, pulmonary smooth muscle and alveolar walls (Brown et al., 2007; Cianciolo et al., 2008). The pet food recall of over 100 brands (listed on the FDA's website) in 2004 and 2007 was one of the largest food safety scares, yet these products only equaled about 1% of the available pet food supply (FDA, 2007c; Turley, 2009).

It was previously speculated that melamine and its related triazines were biotransformed but studies state that these compounds are not metabolized in mammals (Dobson et al., 2008; Filigenzi et al., 2007). Dobson et al. (2008) hypothesized that a mixture of melamine related compounds was responsible for toxicity in cats and dogs. Subsequent studies confirmed that the presence of multiple triazine compounds in foods and feeds caused dangerous health effects in comparison to animals fed individual triazine compounds (Lowy and Pritchard, 2008; Puschner et al., 2007; Reimschuessel et al., 2008). Cats appear to be more susceptible to acute and chronic toxicity than dogs (Brown et al., 2007), especially dogs of larger size (Burns, 2007b). This

observation may be explained by the physiological differences in the tubular function between dogs and cats, an increased number of cats consuming wet foods, and greater food consumption in relation to body size (Brown et al., 2007). Brown et al. (2007) also found that death of cats was generally caused by acute melamine associated renal failure.

On February 6, 2008, the FDA (2009b) announced that two Chinese men and their company, and two men in a U.S. company were indicted by a federal grand jury for their involvement in the deliberate use and importation of contaminated wheat gluten (Olesen, 2010). Their motive for adultering ingredients with melamine was to artificially increase the amount of protein, which is calculated from the amount of nitrogen in a product.

In 2008, melamine was once again found in food production, but this time in contaminated foods for human consumption (Food Report, 2008). Liquid and powdered milk products produced before September 14, 2008 were recalled in China due to an adulteration of milk products where melamine was found in items such as frozen yogurt desserts, biscuits, and confectionary (Food Report, 2008). Twenty-two brands of infant formula in China were found to be contaminated with melamine, with concentrations ranging from 0.1 to 2,563 ppm, and were taken off the market (Ingelfinger, 2008; Li et al., 2009). In response to the contamination, Food Report (2008) published an ad in the October 2008 edition asking for 'scientific experts' from WHO to 'review current knowledge' about melamine and cyanuric acid. The Public Health Agency of Canada (PHAC) publicized a recall on October 28, 2008 that was announced by the Canadian

Food Inspection Agency (PHAC, 2008). The PHAC (2008) urged the public to test plasma creatinine concentrations and perform renal ultrasound on infants that may have been subjected to contamination although no human deaths or illnesses were reported in Canada. Use of infant formulas manufactured in the U.S. was approved by the FDA and as of November 28, 2008 the FDA announced that concentrations ≤ 1 ppm of the individual triazine molecules could be considered safe (Ingelfinger, 2008; Lowy and Pritchard, 2008). The WHO also published guidelines regarding melamine in 2008, where information about melamine and the food recall is outlined. U.S. FDA's interim safety/risk assessment on melamine and its related triazine compounds set the tolerable daily intake (TDI) at 0.63 mg/kg BW/day (FDA, 2009a).

A recent report indicated that 76 tons of melamine contaminated milk powder and dairy products were found in Chinese provinces in July 2010, but this may have been left over products from the 2008 scandal (Olesen, 2010; Reuters, 2010; Wines, 2010). The discovery of these products occurred when an infant formula company tested their product to determine the dilution ratio of the product before marketing (Olesen, 2010). Some of the tainted product had already been included in ice cream and other dairy products but had fortunately not been distributed throughout the market (Olesen, 2010).

Effects on Health: Pathology and Death

Humans

In September 2008, a mass screening of children who may have been exposed to melamine was conducted free of charge by the government of China using guidelines set forth by The Ministry of Health (Liu et al., 2010b). Unknowingly, contaminated infant formula had been distributed throughout China and soon after children were developing atypical health problems. Affected children developed urolithiasis and consequently were easily irritable, would cry during urination, experienced vomiting, polyuria, polydipsia, and, in extreme cases, anuria and even death (Buka et al., 2009). Based on data collected by Ingelfinger (2008), Chinese children exposed to a median level of the infant formula brand containing 2,563 ppm melamine were ingesting 40 to 200 times the TDI. Infants, especially those relying solely on formula as their food source, receive more melamine per kilogram of body weight, making them more susceptible to melamine toxicity (Buka et al., 2009). Children are easily affected by melamine due to their underdeveloped renal system and subsequently, inflammation can cause complete blockage faster than that of a fully grown adult human (Buka et al., 2009).

Soon after the onset of the melamine toxicity outbreak, researchers began collecting and analyzing data related to renal failure among Chinese children. In 2009, He et al. collected data from 15,577 children who had been exposed to adulterated powdered formula. Of these children, 431 had calculi in one kidney and 131 had calculi in both kidneys (He et al., 2009). Seven children had developed ureteric calculi, one had bladder calculi, one had urethral calculi, one had gallbladder calculi and 15 children had urinary tract obstruction (He et al., 2009). Three children were diagnosed with biliary

calculi and upon further investigation, it was determined that these three children had been consuming the most potent powdered formula brand, Sanlu, in which the concentration measured 2,563 ppm, for more than six months (He et al., 2009). Ultrasonography allowed for detection of sand-like microliths that are unique to melamine toxicity; a melamine compound surrounded by uric acid deposits (He et al., 2009). In 2009, Li et al. also collected data regarding the effects of melamine contamination in infants in China. Investigation of those exposed to melamine found that preterm infants, children with previous urinary malformations, children whose parents have a history of urinary tract stones, and children exposed to melamine for long periods of time were more likely to have nephrolithiasis than those not exposed (Li et al., 2009). Li et al. (2009) determined that children exposed to melamine at or below the WHO's recommended safe level of melamine intake of < 0.2 mg/kg/BW/day were still 1.7 times more likely to develop nephrolithiasis than children exposed to no melamine. Research of He et al. (2009) and Li et al. (2009) did not consider the entire population of China. Therefore, the high incidence of melamine contamination may be due to the sampling occurring in a population of low social economic status, where migrant workers are more likely to inadvertently feed their children the low cost, high concentrate melamine contaminated formula.

In 2010, in a follow up study, Liu et al. determined the rate of renal abnormality resolution for affected children in Yuanshi, China (2010b). Fifty-two percent of children recovered from renal failure 48 days after the initial screening and 88% had complete renal recovery after 149 days. These children who had recovered from renal failure

were recovering without any specific medical treatment (Liu et al., 2010b). Liu et al. (2010b) recommends a continuance of this follow up study in order to determine the long term effects on the affected children, especially the 12% that had not fully recovered after six months. Long term effects due to melamine contamination are not understood (Ingelfinger, 2008) and likewise, effects of melamine on unborn children are unknown (CDC, 2008).

Dogs and Cats

Beginning in 1945, melamine was evaluated in animal experimentation. Lipschitz and Stokey (1945) tested the use of melamine in dogs as a diuretic. They found that melamine had diuretic effects in rats and dogs where output of NaCl and water were proportionate to the dose of melamine (Lipschitz and Stokey, 1945). Melamine was excreted in urine partially in the form of crystalline dimelaminemonophosphate and 60 to 86.5% of the melamine was excreted in the urine within 24 hours (Lipschitz and Stokey, 1945). Further investigation of the effects of melamine in dogs was not conducted until the recent outbreaks of melamine toxicity.

Due to the large number of cats and dogs developing renal failure and the high mortality rates, significant data were collected regarding companion animals, especially those who had inadvertently been exposed to melamine contamination. Evaluation of histological effects of melamine associated with toxicity in 2007 confirmed it to be identical to dogs and cats that died in 2004 that had previously been assumed to be a case of mycotoxicosis (Brown et al., 2007).

One of the first cases of melamine occurred when 43 of 70 cats involved in various palatability and acceptability test panels developed clinical signs of toxicity (Cianciolo et al., 2008). Eight of the 70 cats developed feed aversion and 35 developed polydipsia, polyuria, dehydration, vomiting, lethargy, and anorexia, whereas seven of the cats had grossly enlarged kidneys which were discovered upon abdominal palpation (Cianciolo et al., 2008). Upon scrutiny of the ingredients in these trials, researchers found wheat gluten, a thickening agent for wet pet foods such as products containing "gravy," to be a common ingredient in the diet of these sick animals (Burns, 2007a; Dobson et al., 2008; FDA, 2007b). These cats were inadvertently fed melamine contaminated feed. Cats in one group had been fed the contaminated feed for six days, twice daily (Cianciolo et al., 2008). Azotemia, associated with tubular damage and characterized by a marked increase in blood urea nitrogen and creatinine, was found in affected cats (Cianciolo et al., 2008; Puschner et al., 2007). Fifteen out of 19 cats tested positive for azotemia at 7 to 11 days post consumption of the contaminated feed; some reaching levels of BUN > 100 mg/dL and creatinine concentrations > 8 mg/dL (Cianciolo et al., 2008). One cat died and six others had to be euthanized. Of the remaining eight cats, five had recovered from renal failure five weeks after being removed from the contaminated feed (Cianciolo et al., 2008) and the last cat to recover to normal renal function did so at 22 weeks post consumption.

Brown et al. (2007) were able to detect melamine and cyanuric acid at a minimum concentration of 5 ppm in tissues. All dogs and cats in the Brown et al. (2007) study had developed oxalosis and uremia, or elevated blood urea nitrogen, which is a

condition of the blood where concentrations of waste products are higher than normal, indicating renal failure. Of the animals tested, all had marked increases in serum measurements due to melamine exposure: BUN was greater than 130 mg/dL (reference range 20 to 34 mg/dL), serum creatinine reached levels of 7 to 15 mg/dL (reference range 0.9 to 2.1 mg/dL), and hyperphosphatemia was found at 11.3 to 25 mg/dL (reference range 3.2 to 6.2 mg/dL). Animals also developed anorexia, vomiting, lethargy, polyuria, and polydipsia in doses as low as 0.2% of the diet by 12 hours after consumption (Brown et al., 2007; FDA, 2007b; Puschner et al., 2007).

Norsworthy (2008) observed an affected cat that had been treated in 2008 for renal failure after experiencing extreme weight loss. A month after being treated, the cat's creatinine level had increased from 5.9 to 9.7 mg/dL and after an additional four days was measured at a much higher level of 9.9 mg/dL (Norsworthy, 2008). Serum phosphorus concentration was also measured and likewise increased as did the creatinine level (Norsworthy, 2008). The cat's progressively worsening condition soon resulted in euthanization (Norsworthy, 2008).

Another cat diagnosed in 2007 with acute renal failure had been removed from melamine exposure for three subsequent months before being euthanized. Upon necropsy, unique radiating, striated crystals associated with melamine toxicity were detected in distal tubules and collecting ducts of fresh kidney samples (Brown et al., 2007; Reimschuessel et al., 2008). Puschner et al. (2007) found the same amorphous, refractile, and birefringent crystals in fan-shaped to starburst prisms to globular shapes within the distal tubules. Crystals ranged in size, were light green to slightly basophilic,

and visible with and without polarization (Brown et al., 2007). The acidic properties of urine in cats and dogs may account for the development of insoluble melamine-cyanurate crystals (Puschner et al., 2007). Cats exhibited greater susceptibility to lesions with increasing dosage levels, therefore cats fed high combinations of melamine and cyanuric acid had more severe kidney lesions (Puschner et al., 2007). Puschner et al. (2007) confirmed that dietary exposure of melamine to cats can lead to toxicity and acute renal failure can result from exposure to doses as low as 32 mg/kg of body weight.

Upon further investigation of the urine sediment, Cianciolo et al. (2008) and Puschner et al. (2007) both found microscopic, green-brown circular crystals. The crystals ranged in size; most measured 15 to 20 μm in diameter and had two distinct concentric rings with linear striations that gave the appearance of spokes radiating from the center and other crystals were smaller, 2 to 3 μm in diameter, with single-to-multiple refractile granules (Cianciolo et al., 2008). These crystals were also found in cats fed a combination of 1% melamine and 1% cyanuric acid in numbers as great as 50 crystals per 1 cm-diameter section of the kidney (Puschner et al., 2007). Crystals were frequently associated with sloughed epithelial cells and hypertrophied endothelial cells, causing perivascular inflammation in some cats (Cianciolo et al., 2008). Tubular necrosis and crystalluria were observed in multiple cats, and one cat had developed a hematoma (Cianciolo et al., 2008). Cianciolo et al. (2008) also found that older cats were more likely to die of melamine toxicity than young cats.

Cattle

Melamine degradation results in analogs of ammeline, ammelide, cyanuric acid, biuret, and eventually urea (Jutzi et al., 1982; Figure 2.3). Biuret and urea have been included in cattle diets to offer the rumen a non-protein nitrogen source for microbial protein synthesis. Newton and Utley (1978) conducted an experiment with cattle testing the availability and use of melamine as a non-protein nitrogen source. Melamine inclusion at 0.9% of the diet was deemed unsuccessful in providing an adequate level of ammonia that is necessary for maximizing microbial protein synthesis in cattle ($P > 0.05$; Newton and Utley, 1978). Feed refusal of melamine diets was fairly consistent among 2/3 of the cattle in the study (Newton and Utley, 1978). Cattle that continued consumption of the contaminated diet were able to digest the melamine nitrogen equal to that of cottonseed meal (which was used as a comparative non-protein nitrogen source), but more of the nitrogen was excreted in the urine ($P < 0.06$; Newton and Utley, 1978). This excretion of melamine nitrogen was more non-ammonia, non-urea nitrogen than that which was excreted by steers fed cottonseed meal ($P < 0.01$) or urea ($P < 0.05$; Newton and Utley, 1978). Newton and Utley (1978) hypothesized that the nitrogen in the melamine ring may be absorbed but cannot be utilized for protein synthesis.

Another study, by Shen et al. (2010), studied melamine transfer in milk of dairy cows. After 2 days of being fed melamine, residues of melamine could be found in the milk of cattle, but at four days after contaminated feed removal melamine was not detectable in milk (Shen et al., 2010). Milk melamine concentration increased linearly as melamine intake increased and a level of intake equaling < 29.6 mg/600 kg cow would

maintain a milk melamine level below the 0.02 mg/kg LOD (Shen et al., 2010). Intake exceeding 312.7 mg of melamine would result in a product unsafe for use in infant formula and melamine fed ≥ 715.1 mg would result in a product unsuitable for use in milk powder production (Shen et al., 2010). Moreover, it was discovered that milk melamine concentration was dependent on dose size but not significantly influenced by milk yield, although transfer efficiency from feed to milk was linearly related to milk yield (Shen et al., 2010).

Rats and Mice

Mice and rats have been used in several melamine and/or cyanuric acid studies. Information from these studies has been used by FDA, WHO (2008), and other health organizations to determine toxicity. Melnick et al. (1984) fed rats and mice contaminated diets and upon investigation found a high number of animals with uroliths. Uroliths were more prevalent in the male species in both rats and mice, and male rats also developed higher susceptibility to urothelial cell carcinomas in the bladder when fed 4,500 ppm melamine (Melnick et al., 1984). Ogasawara et al. (1995) also discovered lesions in the urinary tract of rats which were caused by melamine calculi irritating the bladder epithelium. Comparable to other species, when individual triazines were fed to rats no toxicity was observed, suggesting that the individual compounds were not precipitating in the kidney (Dobson et al., 2008). When the triazines were combined, melamine, ammeline, ammelide, and cyanuric acid in a 400/40/40/40 mg/kg/day mixture and another of melamine and cyanuric acid at 400/400 mg/kg/day, both combinations were considered toxic (Dobson et al., 2008).

After three days of consumption of either triazine combination, rats became oliguric, hematuric, and had decreased food consumption and body weight (Dobson et al., 2008), although renal failure progression differed among individual rats.

Research by Mast et al. (1983) calculated a clearance rate of melamine equal to 2.7 hours in rats. Through urination, rats eliminated 90% of the melamine they had consumed within 24 hours (Mast et al., 1983). Crystal formation in kidneys of rats exposed to melamine and cyanuric acid was an exact replica of crystals isolated from the melamine-cyanuric acid contaminated wheat gluten (Dobson et al., 2008). Additionally, in order to determine the transfer rate an experiment was conducted by Jingbin et al. (2010). Jingbin et al. (2010) administered melamine at 400 mg/kg BW to pregnant rat dams and 1,117.7 ± 304.4 μg/g melamine was detected in the placenta. Plasma concentration of uric acid and creatinine were higher than that of control rats (Jingbin et al., 2010). Mortality was also significant in pregnant rats fed melamine and cyanuric acid in combinations as low as 40/40 mg/kg/day (Jingbin et al., 2010). Jingbin et al. (2010) concluded that pregnant dams consuming melamine alone in high concentration or for long periods of time will indirectly cause exposure of melamine to the dams' fetuses so expecting mothers should avoid contact with melamine in order to prevent indirectly contaminating their fetus.

Fish

In a study of melamine toxicity in fish, Reimschuessel et al. (2008) found that trout, salmon, and catfish fed melamine and cyanuric acid combinations excreted white feces, although most fish did not experience renal failure due to the nitrogenous wastes

generally being excreted by way of the gills. Observation of histological sections presented gold-brown needle-like crystals that were forming spheroid aggregates in the kidneys of fish fed melamine and cyanuric acid combinations and were evident up to 14 days post melamine/cyanuric acid consumption (Reimschuessel et al., 2008). Reimschuessel et al. (2008) also tested the combination of melamine and cyanuric acid in one treatment and in another treatment fed melamine first, followed six days later by cyanuric acid. This test yielded similar results among the treatments, with both treatments causing development of similar renal crystals in the fish. For the fish fed cyanuric acid alone, two out of the six trout had pale gold crystals forming in the renal tissues that were distinguishable from the crystals formed by combinations of cyanuric acid and melamine (Reimschuessel et al., 2008).

Muscle tissue in fish had higher residues of melamine and cyanuric acid when individual triazines were fed, which is hypothesized by Reimschuessel et al. (2008) to correlate to the idea that a melamine-cyanurate complex precipitates in the GI tract and kidneys. This precipitation is caused by the loss of bioavailability of the complex due, in large part, to the low water solubility of the compound (Tyan et al., 2009). Furthermore, melamine concentrations in fish tissue were higher than cyanuric acid concentrations although both triazines were administered individually at equal doses (Reimschuessel et al., 2008). Two fish in the Reimschuessel et al. (2008) study died due to toxicity of melamine and cyanuric acid combination, but no mortality was observed in treatments receiving individual triazines.

Pigs

Research performed by Reimschuessel et al. (2008) evaluated the effects of melamine on pigs. The treatments consisted of a control pig, a pig fed 400 mg/kg of melamine alone, a pig fed 400 mg/kg of cyanuric acid alone, and one pig fed 400 mg/kg of melamine and cyanuric acid in combination. In the study, they found significant pathological effects: edema of the fascia surrounding the kidneys of the pig fed the 1:1 melamine and cyanuric acid combination, and kidneys with pale, mottled, grayish tan color that were covered in small red foci. Elevated creatinine and BUN levels were detected in the serum of the pig fed melamine and cyanuric acid, but were not different from the control, melamine alone, or cyanuric acid alone pig ($P > 0.05$; Reimschuessel et al., 2008).

Melamine administered intravenously in a study by Baynes et al. (2008), was performed to determine the elimination rate of melamine in the pig model. This study determined that melamine had a half-life of 4.04 (± 0.37) hours and clearance of 0.11 (± 0.01) L/h/kg (Baynes et al., 2008). Research by Mast et al. (1983) reported clearance equaling 2.7 hours in rats. Because of the rapid elimination of melamine, it can be hypothesized that accumulation of melamine in the animal body is highly improbable. Distribution of melamine may be largely limited to the extracellular fluid, therefore reducing absorption into most organs (Baynes et al., 2008). In contrast to the rat clearance study, pigs have a clearance rate almost five times less than that of rats (Mast et al., 1983). Consequently, Baynes et al. (2008) calculated that 99% of melamine administered should be cleared from a pig's body within 28 hours from exposure, leaving the animal safe for human consumption.

Poultry

Effect of melamine on avian species was tested in broilers by Lü et al. in 2009. The research was conducted to determine growth performance, serum concentrations, and residual effects. Exposure to melamine up to 1,000 mg/kg of diet for 42 consecutive days did not cause toxicity in broilers (Lü et al., 2009). Weight gain, feed intake, feed conversion (feed:gain) and mortality of broilers fed levels of melamine up to 1,000 mg/kg of diet did not differ from controls ($P > 0.05$; Lü et al., 2009). Diets contaminated with ≤ 50 mg/kg of melamine had residual levels in tissues lower than the LOD at day 28 and 42 (Lü et al., 2009). Doses of melamine > 50 mg/kg resulted in higher melamine residue levels in the kidneys and lower residue levels in the breast muscle and with increased melamine exposure there was an increase in residue levels in tissues ($P < 0.05$; Lü et al., 2009). Day 42 of melamine exposure resulted in lower melamine concentration in tissues than that of day 28, which suggests that broilers are better able to clear melamine from their body as they age (Lü et al., 2009). After a seven-day withdrawal period from contaminated feed, tissues were found to be depleted of any residual melamine (Lü et al., 2009).

Summary

Dobson et al. (2008) hypothesized that melamine-cyanurate compounds are dissociated in the gastrointestinal tract due to the low pH, later absorbed in different areas of the gastrointestinal tract and are then reformed in the kidney tubules. Generally, clearance of melamine is completed primarily by way of renal filtration due to

the molecule being polar and small (Baynes et al., 2008), but combinations of melamine and cyanuric acid are not easily excreted. Cyanuric acid altered elimination kinetics of melamine, causing crystals to precipitate in renal tubules so the compounds could not be eliminated from the body (Cianciolo et al., 2008).

Waste products in the blood are not cleared as readily in animals diagnosed with renal problems. Increased BUN and creatinine are indicative of renal failure (Brown et al., 2007; Cianciolo et al.; Dobson et al., 2008). Dobson et al. (2008) found that uric acid is not effectively filtered out of the body, and may provide areas of attachment for the melamine and cyanuric acid compounds in the renal system. Formation of crystals in the renal tubules causes inflammation and blockage and subsequently causes a significant increase in kidney size (Dobson et al., 2008). The medulla of the kidney has been found to collect more crystals than other areas of the kidney (Dobson et al., 2008; Puschner et al., 2007; Reimschuessel et al., 2008). The distal tubules and loops of Henle were found to be dilated in affected animals (Dobson et al., 2008), and contained necrotic cells with basophilic epithelium (Reimschuessel et al., 2008).

"One part per million is a very low level of melamine-if no other chemical is included" Lin tells WebMD (DeNoon, 2008). "But if you have one part per million of melamine and 1 part per million of cyanuric acid, that is a big problem. The two chemicals react to form crystals, and that can be a big risk, especially for infants" (DeNoon, 2008). Melamine was found to be excreted from the body in 3 to 4 hours and accumulation of melamine in the body is not expected if administered in small amounts

(DeNoon, 2008; Tyan et al., 2009). Fluid therapy has been effective in restoring health in animals developing renal failure (Burns, 2007a).

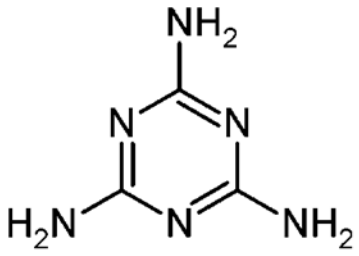

Figure 2.1. Melamine structure

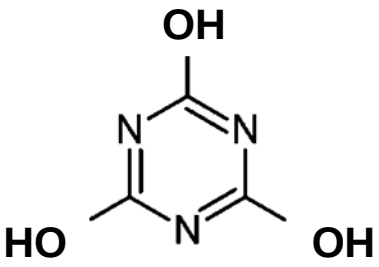

Figure 2.2. Cyanuric acid structure

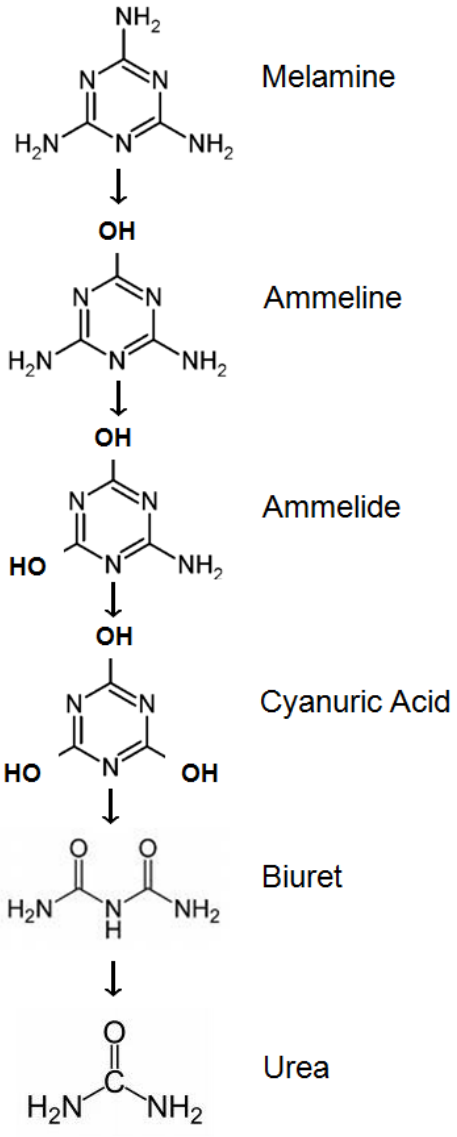

Figure 2.3. Degradation of the melamine structure to urea

CHAPTER 3

Effects of Melamine in Young Broiler Chicks

Introduction

There have been recent concerns regarding adulteration of protein ingredients, used in pet foods and human foods, with melamine (M). Melamine (1, 3, 5-triazine-2, 4, 6-triamine) is a white, crystalline compound with a molecular formula of $C_3H_6N_6$ (NIOSH, 2006; OSHA, 2009; Safety Data, 2008). Melamine is typically used in industry as a synthetic chemical for production of resins, foams, cleaning products, fertilizer (although prohibited from use in the USA as a fertilizer), pesticides and fire retardant materials (FDA, 2007a; Lowy and Pritchard, 2008). More recently, melamine has been found in ingredients fed to animals and humans. Methods such as the Kjeldahl method or the Dumas method are used to calculate the amount of protein in an ingredient or diet based off of the amount of nitrogen in the ingredients. The amount of nitrogen in the sample is then multiplied by 6.25 to determine protein content. Because melamine contains 66% nitrogen by mass, these methods indicate melamine to be rich in protein, which implies that this product would be useful in increasing protein in a diet. Unfortunately, these methods can potentially overestimate the amount of protein in nitrogen rich, yet nutritionally inadequate compounds such as melamine (Ingelfinger, 2008).

On March 16th 2007, Menu Foods placed a voluntary recall on wheat gluten and rice protein products that were imported from the Chinese food company, ChemNutra (Burns, 2007a; FDA 2007b). Ingredients contaminated with melamine, including pet food, fish feed and vegetable proteins, were instructed to be recalled and destroyed (FDA, 2007b). The Food and Drug Administration (2007c) listed over 100 brands involved in the pet food recall, making it one of the largest pet food safety problems in the United States (Turley, 2009). From April 5 to June 6, 2007, 235 cats (61% mortality) and 112 dogs (74% mortality) were diagnosed with "pet food-induced nephrotoxicity" based on high concentrations of blood urea nitrogen and creatinine (Burns, 2007b). The formation of "spoke-like" crystals in kidneys of affected animals was suspected of compromising kidney function, leading to acute renal failure and death in cats and dogs. Animals also developed lesions including oral ulcers along the tongue and mineralization in the gastric mucosa, pulmonary smooth muscle, and alveolar walls (Brown et al., 2007; Cianciolo et al., 2008).

Furthermore, pigs and chickens consuming melamine contaminated pet food byproducts were traced to various states across the nation (Burns, 2007a; FDA, 2007a). Research by Filigenzi et al. (2007) determined that melamine residues in the tissue of the affected animals were not high enough to pose a threat to human health. Upon further investigation, it was determined that melamine and its related triazine compounds are generally excreted via the kidneys and are not metabolized in mammals (Dobson et al., 2008; Filigenzi et al., 2007).

The objective of this study was to determine if melamine will cause toxic effects in young chicks, and if so, at what dose of melamine will toxicity occur.

Materials and Methods

Diet Preparation

Seven dietary treatments were prepared using graded levels of melamine purchased from Sigma-Aldrich Chemical Company (St. Louis, Mo.). Melamine was included at 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0% to a basal corn-soybean meal diet (Table 3.1) that was formulated to meet or exceed requirements of broiler chicks as recommended by the National Research Council (NRC, 1994). Melamine was substituted for sand to obtain the desired dietary melamine concentrations.

Birds, Management and Response Variables

One hundred and seventy five day-old male Ross broiler chicks were purchased from a commercial hatchery. Chicks were weighed, wing-banded, and sorted to a randomized block design in stainless steel battery pens. Housing was in an environmentally controlled room with a 24-hour constant light schedule. Feed and water were supplied for *ad libitum* consumption. Day-old chicks were divided among seven dietary treatments (five birds per pen and five replicate pens per treatment) and fed dietary treatments for 21 days. The animal care and use protocol was reviewed and approved by the University of Missouri Animal Care and Use Committee (ACUC).

On day 21, chicks were euthanized using carbon dioxide, weighed, and serum was collected from three birds per pen. Chicks that died before termination were

weighed and sent to a pathology lab for necropsy. Residual feed was measured and recorded. At termination, serum, kidney, liver, bile, and breast muscle samples were collected from three birds per pen, and frozen for further analysis. Response variables included feed intake, body weight gain, feed conversion (feed:gain), mortality, serum chemistry, relative kidney and liver weights, gross and histopathology of kidneys, and melamine concentrations in breast muscle, kidney, liver, and bile. Five sections of spleen, thymus, bursa of Fabricius, liver, kidney, trachea, proventriculus, heart, and jejunum were collected per treatment and analyzed for pathological effects.

Melamine Analysis

Extraction of melamine (Fisher Scientific Inc., Pittsburgh, PA, USA) in samples was based upon a standard FDA method for melamine detection with some modifications (FDA, 2007). Briefly, 50% (v/v) acetonitrile in water was used to extract melamine from all samples. For feed samples, melamine was extracted at a ratio of 0.1 g sample to 10 mL extraction solvent. The samples were then sonicated using an ultrasonic processor equipped with a 6.5 mm tapered microtip (Sonics & Materials, Inc. Newtown, CT) for 2 min with 30 s working and 30 s interval at an amplitude of 36%. For bile samples, melamine was extracted at a ratio of 0.5 mL sample to 0.5 mL extraction solvent. For breast muscle, liver, and kidney samples, melamine was extracted at a ratio of 2.0 g sample to 15 mL extraction solvent, and homogenized for 1 min. All extracts were centrifuged at 2,266 x g for 5 to 20 min, and the supernatant from each sample was collected. Extracts of breast muscle, liver, and kidney samples, were defatted using methylene chloride (extract: methylene chloride = 1:1, v/v). All extracts were then

diluted with a stock solution of 0.1 N HCl and filtered through a 0.45 µm nylon syringe filter. A melamine standard stock solution (1.0 mg/mL) was prepared with a acetonitrile:water (60:40 v/v). A series of concentrations of standard melamine solutions were prepared by diluting the stock solution with 0.1 N HCL to obtain melamine concentrations of 1, 5, 10, 25, 50, 75, 100, 200, 300, and 400 µg/mL, respectively.

For the HPLC analysis, an Agilent 1100 series HPLC system with a 1200 series automatic injector was used. The system consisted of a quaternary pump, a degasser, a column oven, and a diode array detector. The mobile phase was an 85:15 (v/v) buffer containing 10 mM citric acid and 10 mM sodium octanesulfonate (pH 3.0): acetonitrile. Test conditions included: Zorbax SB-C8 (4.6 mm x 75 mm, 3.5 µm particle, Agilent) column; column temperature of 40°C; flow rate of 1.0 mL/min; DAD spectra, 190 to 400 nm, detected at 240 nm.

For HPLC data analysis, a melamine standard curve was obtained by establishing a plot correlating the concentrations of standards to the peak areas. Melamine in samples was confirmed by retention time and a specific absorbance spectrum with a λmax at 236 nm. Melamine contents in samples were quantified by plotting peak areas into the standard curve.

Statistical Analyses

Data for all response variables were subjected to regression analysis using the general linear models procedure of SAS (SAS Institute, 2006). Regression analysis best fit the means relative to a linear ($y_i = a + bx_i + E_i$) and/or quadratic ($y_i = a + bx_i + cx^2_i + E_i$)

response. Dunnett's test was run to calculate means among treatments and determine at which level of inclusion the specific parameter was significant from the control. Statistical significance was accepted at a p-value of < 0.05.

Results

Mortality and Growth Performance

The effects of dietary treatments on mortality and growth performance are summarized in Table 3.2. Mortality increased quadratically ($P < 0.0004$) with increasing dietary concentrations of melamine. However, compared with controls, mortality was only higher ($P < 0.0001$) in birds fed ≥ 2.5% melamine. Feed intake decreased linearly ($P < 0.0001$), whereas body weight gain decreased quadratically ($P < 0.0131$) with increasing dietary concentrations of melamine. Compared with controls, both feed intake and body weight gain were only lower ($P < 0.0001$) in birds fed ≥ 1.0% melamine. The ability of birds to convert feed to body weight gain (F:G) decreased quadratically ($P < 0.0098$) with increasing dietary concentrations of melamine. However, compared with controls, F:G was only higher ($P < 0.0001$) in birds fed ≥ 2.5% melamine.

Organ Weights

Effects of dietary treatments on relative kidney and liver weights are summarized in Table 3.2. Relative kidney weights increased linearly ($P < 0.0001$), whereas relative liver weights increased quadratically ($P < 0.0453$) with increasing dietary concentrations of melamine. Compared with controls, relative kidney weights

were only higher ($P < 0.0001$) in birds fed ≥ 1.5% melamine, whereas relative liver weights were only higher in birds fed ≥ 2.5% melamine.

Tissue Residues

Table 3.3 contains a summary of the effects of dietary treatments on melamine residues in breast muscle, liver, kidney, and bile. Melamine residues in breast muscle and liver tissue increased linearly ($P < 0.0001$) with increasing dietary concentrations of melamine, whereas melamine residues in kidney and bile increased quadratically ($P < 0.0151$) with increasing dietary concentrations of melamine (Table 3.3). Compared with controls, melamine concentrations in liver and kidney were higher ($P < 0.0001$) in birds fed all levels of melamine, whereas melamine concentrations in breast muscle and bile were only higher in birds fed ≥ 1.0% melamine ($P < 0.0001$). Melamine residues, averaged across all dietary treatments, were highest in the kidney (580 ppm), intermediate in the liver (387 ppm) and bile (392 ppm), and lowest in breast muscle (326 ppm).

Serum Chemistry

Effects of dietary treatments on serum chemistries are summarized in Table 3.4. Values for serum glucose of birds fed 0.5% melamine were increased compared to controls but serum glucose values returned to control values in all other treatments containing melamine. Values for serum albumin, total protein, globulin, and calcium were increased quadratically in birds as percent of dietary melamine increased, and became significantly higher compared to controls in birds fed 3.0% melamine. Values for serum aspartate transaminase, gamma gluatamyltransferase and uric acid increased

linearly with increasing levels of melamine in the diet, however the values were only significantly higher than controls in birds fed 3.0% melamine for AST and GGT.

Pathology

Gross Pathology - Early Mortality

The gross pathology of broilers fed 2.0, 2.5 and 3.0% melamine that died early in the experiment were uniform. The birds had enlarged, pale kidneys, and microscopic examination of kidney tissue revealed numerous spherical, crystalline particles that were singe (2 to 20 micron diameter) or aggregated into a larger mass (Figure 3.1). The gallbladder contained brown to green bile that was opaque rather than clear (Figure 3.2). Microscopic examination of the bile (two drops of bile on a slide with a coverslip) revealed numerous spherical, smooth brown crystalline particles that were single (2 to 20 micron diameter) or aggregated into a larger mass (Figure 3.3). No gross lesions were observed in the livers of these birds.

Histopathology - Early Mortality

Renal pathology was significant in all kidney sections examined. In the seven broilers that died during the first five days of treatment, histopathology included a severe accumulation of eosinophilic to basophilic casts in the collecting ducts and tubules with an associated moderate heterophil infiltration of the collecting ducts and tubules. The tubular cases were composed of what appeared to be single to aggregated spherical crystalline structures (Figure 3.4). Inflammation of the kidney was found to be significant in affected birds (Figure 3.5). In birds that died after five days of treatment, no inflammation was noted in the kidney sections and a severe accumulation of

eosinophilic to basophilic casts in the collecting ducts and tubules was the primary lesion. The tubular cases were composed of what appeared to be single to aggregated spherical crystalline structures that were not polarizable when examined under polarized light.

Gross Pathology - Termination of Experiment

From all treatments, gross examination of broilers that survived to the end of the study revealed clear size differences among treatments which are best characterized by the body weight data. Gross examinations of the broilers were otherwise unremarkable and all tissues, including the liver and kidney, appeared normal.

Histopathology - Termination of Experiment

Histopathology of the liver from all treatments and the kidney of birds fed 0, 0.5, and 1.0% melamine were unremarkable. In birds fed 1.5% melamine, one kidney section had a mild accumulation of spherical basophilic crystals in the collecting ducts and collecting tubules, whereas five kidney sections were unremarkable. In birds fed 2.0% melamine, two kidney sections had a mild accumulation of spherical eosinophilic or basophilic crystals in the collecting ducts and collecting tubules, whereas four kidney sections were unremarkable. In birds fed 2.5% melamine a variety of mild changes were noted. Two kidney sections had eosinophilic casts in the lumen of embryonal nephron tubules. One kidney section had both heterophil and eosinophilic cases within tubules of the embryonal nephrons, another kidney section had eosinophilic casts within collecting tubules and heterophils within the collecting ducts, and two kidney sections were unremarkable. A variety of mild lesions were noted in birds fed 3.0% melamine.

One kidney section had both eosinophilic casts and heterophils in the collecting tubules with eosinophilic casts within embryonal nephrons. Two kidney sections had isolated eosinophilic casts in the embryonal nephrons and collecting tubules. One kidney section had mineralized basophilic casts and eosinophilic casts within the collecting tubules and collecting ducts. Two kidney sections were unremarkable.

A broad selection of tissues were collected for histopathology from broilers fed no melamine (3; Control) and broilers fed 3.0% melamine (5). In birds fed the control diet with no melamine, sections of spleen, thymus, bursa of Fabricius, liver, kidney, trachea, proventriculus, heart, and jejunum were unremarkable. In birds fed 3.0% melamine, mild kidney lesions were noted as described above. A moderate depletion of medullary lymphocytes was noted in all sections of the bursa of Fabricius. Sections of spleen, thymus, liver, trachea, proventriculus, heart, and jejunum were unremarkable.

Discussion

In this study, mortality was 16 to 36% in birds fed ≥ 2.0% melamine, however 75% of this mortality occurred by day 10. These results suggest that older birds are less sensitive to melamine or that surviving birds were able to more efficiently excrete melamine. Lu et al. (2009) reported that tissue melamine residues were lower in birds fed melamine for 42 days compared to those fed melamine for 28 days and hypothesized that broilers may have developed more capacity to clear melamine from body tissues with advancing age. In the only other study found in the literature (Lu et al., 2009) in which melamine was fed to broilers, concentrations of melamine up to 0.1% of the diet did not cause mortality. Significant mortality has been reported previously in

cats and dogs consuming food contaminated with both melamine and cyanuric acid, and the mortality was attributed to renal failure in these species (Brown et al., 2007; Cianciolo et al., 2008). Renal failure was also considered to be the cause of mortality in the present study in which birds were fed only melamine, and was also identified in rats fed combinations of melamine and cyanuric acid (Dobson et al., 2008). These data indicate, at least in birds, that high dietary concentrations of melamine alone will cause mortality due to renal failure.

In the present study, growth performance of birds was not negatively affected until birds were fed ≥ 1.0% melamine. These results are consistent with a previous study by Lu et al. (2009) in which growth performance was not negatively affected in broilers fed up to 0.1% melamine for 42 days. Decreased feed intake and growth have also been reported in cats and rats consuming melamine/cyanuric acid contaminated food (Dobson et al., 2008; Cianciolo et al., 2008).

Increases in relative kidney (≥ 1.5% melamine) weights observed in the present study have been observed previously in cats (Cianciolo et al., 2008) and rats (Dobson et al., 2008) fed food containing melamine and cyanuric acid, and are consistent with the kidney being the target organ of melamine/cyanuric acid. In contrast to the present study, in which increases in the relative weights of liver was observed, changes in liver weights were not observed in cats (Cianciolo et al., 2008) or rats (Dobson et al., 2008) fed melamine and cyanuric acid.

Tissue melamine residue results indicated that melamine residues were highest in the kidney (391 ppm), intermediate in the liver (387 ppm), and lowest in breast

muscle (326 ppm). These results are consistent with a previous report by Lü et al. (2009) who also observed a similar tissue distribution in broilers fed lower levels of melamine (0 to 0.1%). The higher melamine concentrations in the kidney are also consistent with previous reports indicating that the kidney is the target organ for melamine toxicity and where the formation of melamine/cyanuric acid crystals occur (Brown et al., 2007; Cianciolo et al., 2008; Dobson et al., 2008; Ingelfinger, 2008). Additionally, Reimschuessel et al. (2008) hypothesized that residue levels are higher in animals fed individual triazines than those fed combinations due to a melamine-cyanurate complex precipitating in the gastrointestinal tract. While significant levels of melamine in tissues were observed in this study, the residue levels were severely diluted compared to the concentrations that had been consumed; birds consuming 5,000 ppm melamine had 151 ppm melamine residue reported in the kidney. This is supported by Baynes et al. (2008) who hypothesized that melamine distribution may be largely limited to extracellular fluid, reducing absorption into most organs.

The increase in bile melamine concentrations observed in the present study has not been reported previously, suggesting one of two possibilities: bile was not evaluated in previous studies or biliary excretion does not occur in the species evaluated (primarily mammals). It may well be that biliary excretion is a normal route of excretion in birds or that biliary excretion is an additional route of excretion in birds fed high concentrations of melamine.

Changes in serum values may suggest that the kidney and/or liver were less efficient in removing waste products from the blood, but because all serum values for

this experiment fell within or near normal expected values for poultry species (Braun et al., 1982; Coles, 1985 and 1997; and Puls, 1994), this assumption can not be made based on these results alone.

Renal lesions and crystals observed in the present study were similar to those observed within the renal tubules of cats, dogs, and rats consuming melamine and cyanuric acid and experiencing renal failure (Brown et al., 2007 and Cianciolo et al., 2008; Dobson et al., 2008). The kidney histological results in the present study were similar to histology results of rats fed a combination of 400 mg/kg/day of dietary melamine and 400 mg/kg/day of cyanuric acid (Dobson et al., 2008) and cats (Brown et al., 2007), dogs (Cianciolo et al., 2008), and fish (Reimschuessel et al. (2008) fed food containing both melamine and cyanuric acid. Mortality observed in studies with cats, dogs, and rats was attributed to deposits of melamine/cyanuric acid crystals that block renal tubules leading to renal failure. Likewise, deposits of melamine crystals appear to be responsible for the renal failure and mortality observed in the present study. The nature of melamine crystals in this study was different from melamine/cyanuric acid crystals observed by Brown et al. in 2007, as they were nonpolarizable when examined under polarized light.

Results of this study indicate that melamine at concentrations greater than or equal to 1.0% is toxic to broilers fed dietary treatments from hatch to day 21. Additionally, broilers can tolerate up to 5,000 mg melamine/kg (0.5%) diet with no adverse effects on growth performance but at this dietary concentration kidney and liver contain significant concentrations of melamine. Although mortality was not

significant in birds fed up to 2.0% melamine, melamine residue concentrations could potentially exceed what is considered safe by the FDA for human consumption (FDA, 2009a). Birds fed greater than or equal to 1.0% melamine may contain enough melamine residue to exceed the tolerable daily intake (TDI) of 0.63 mg/kg BW/day, assuming a 63 kg person consumes 454 grams of melamine contaminated breast muscle in one day. Because the concentrations fed in this study were considerably higher than what was fed inadvertently to companion animals and furthermore livestock animals in 2007, this study confirms that consumption of the affected pigs and chickens generally would not cause harm to human health (Filigenzi et al., 2007).

Table 3.1. Ingredient and nutrient composition of basal ration

Item	Composition (%)
Ingredient	
Corn	55.45
Soybean Meal	30.25
Pork Meal	4.74
Corn Oil	4.21
Fish Meal	3.68
Dicalcium	0.36
Limestone	0.47
Salt	0.35
Methionine	0.19
Trace Mineral[1]	0.11
Vitamin Mix[2]	0.08
Selenium Mix[3]	0.06
Lysine	0.04
Copper Sulfate	0.00
Sand	3.00
Total	100
Nutrient composition (calculated)	
Crude Protein (%)	23.00
Metabolizable Energy (Kcal/kg)	3200
Lysine (%)	1.30
Methionine (%)	0.55
Methionine + Cysteine (%)	0.90
Threonine (%)	0.85
Calcium (%)	1.00
Phosphorus (% Av.)	0.45

[1]Trace mineral mix provided (mg/kg of diet): manganese, 110 mg from $MnSO_4$; iron, 60 mg from $FeSO_4 \bullet 7H_2O$; zinc, 110 mg from $ZnSO_4$; iodine, 2 mg from ethylenediamine dihydroidodide.
[2]Vitamin mix supplied (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.
[3]Selenium premix provided 0.2 mg of Se/kg of diet from $NaSeO_3$.

Table 3.2. Effects of melamine on performance of broiler chicks[1]

	Treatments[2] – Melamine %							ANOVA[5]		Regression[6]	
Item	0	0.5	1.0	1.5	2.0	2.5	3.0	Pooled SEM	P-value	L/Q	P-value
FI, g	910	884	811*	787	645	590	523	26	<.0001	L	<.0001
BWG, g	750	751	682*	608	533	446	382	17	<.0001	Q	0.0131
F:G	1.199	1.177	1.189	1.297	1.248	1.404*	1.551	0.049	<.0001	Q	0.0098
Organ Weight[3]											
Kidney	0.87	0.90	0.97	1.04*	1.07	1.18	1.24	0.07	<.0001	L	<.0001
Liver	3.17	3.28	3.08	3.44	3.39	3.70*	3.95	0.22	<.0001	Q	0.0453
Mort[4] %	4	0	0	0	16	20*	36	4	<.0001	Q	0.0004

[1]Data are means of 25 birds per treatment for growth performance parameters and 15 birds per treatment for organ weights.
[2]0 = Basal diet (BD); 0.5 = BD + 0.5% M; 1.0 = BD + 1.0% M; 1.5 = BD + 1.5% M; 2.0 = BD + 2.0% M; 2.5 = BD + 2.5% M; 3.0 = BD + 3.0% M.
[3]Relative organ weights expressed as a percentage of body weight.
[4]Mort = Mortality %
[5]One way analysis of variance values
[6]Regression- L = linear regression and Q = quadratic regression
*Signifies level at which melamine became toxic for specific parameters in comparison to control birds.

Table 3.3. Residue levels of melamine in breast muscle, liver, kidney, and bile of broiler chicks[1]

	Treatments[2] – Melamine %							ANOVA[3]		Regression[4]	
Item	0	0.5	1.0	1.5	2.0	2.5	3.0	Pooled SEM	P-value	L/Q	P-value
Breast muscle	0	99	212*	358	455	557	601	40	<.0001	L	<.0001
Liver	0	151*	339	353	576	590	701	45	<.0001	L	<.0001
Kidney	0	257*	499	554	899	846	992	40	<.0001	Q	0.0151
Bile	68	96	225*	450	613	670	615	6	<.0001	Q	<.0001

[1] Melamine residual levels in breast muscle (5 birds per treatment), liver (5 birds per treatment), kidney (4 birds per treatment), and bile (2 birds per treatment) are measured in ppm.
[2]0 = Basal diet (BD); 0.5 = BD + 0.5% M; 1.0 = BD + 1.0% M; 1.5 = BD + 1.5% M; 2.0 = BD + 2.0% M; 2.5 = BD + 2.5% M; 3.0 = BD + 3.0% M.
[3]One way analysis of variance values
[4]Regression- L = linear regression and Q = quadratic regression
*Signifies level at which melamine became toxic for specific parameters in comparison to control birds.

Table 3.4. Serum chemistries of broiler chicks fed graded levels of melamine[1]

	Treatments[2] – Melamine %							ANOVA[4]		Regression[5]	
Item[3]	0	0.5	1.0	1.5	2.0	2.5	3.0	Pooled SEM	P-value	L/Q	P-value
GLU (mg/dL)	343	406*	360	370	373	382	380	43	0.0118	-	0.5857
ALB (g/dL)	1.04	1.04	1.05	1.01	1.04	1.15	1.22*	0.08	0.0227	Q	0.0129
TP (g/dL)	2.41	2.36	2.37	2.25	2.39	2.55	2.79*	0.17	0.0126	Q	0.0025
GLOB (g/dL)	1.37	1.32	1.31	1.24	1.35	1.40	1.57*	0.10	0.0089	Q	0.0016
CA (mg/dL)	10.83	11.09	10.83	10.55	11.19	11.31	11.72*	0.34	0.0169	Q	0.0151
AST (IU/L)	188	219	223	217	250	257	354*	53	0.0372	L	0.0004
GGT (U/L)	12.53	11.47	13.87	14.53	12.53	14.00	15.67*	1.43	0.0102	L	0.0046
UA (mg/dL)	7.45	9.27	6.63	8.40	9.37	10.34	8.91	1.65	0.2792	L	0.0690

[1]Data are means of 15 birds per treatment.
[2]0 = Basal diet (BD); 0.5 = BD + 0.5% M; 1.0 = BD + 1.0% M; 1.5 = BD + 1.5% M; 2.0 = BD + 2.0% M; 2.5 = BD + 2.5% M; 3.0 = BD + 3.0% M.
[3]GLU = glucose, ALB = albumin, TP = total protein, GLOB = globulin, CA = calcium, AST = aspartate transaminase, GGT = gamma glutamyltransferase, UA = uric acid.
[4]One way analysis of variance values
[5]Regression- L = linear regression and Q = quadratic regression
*Signifies level at which melamine became toxic for specific parameters in comparison to control birds.

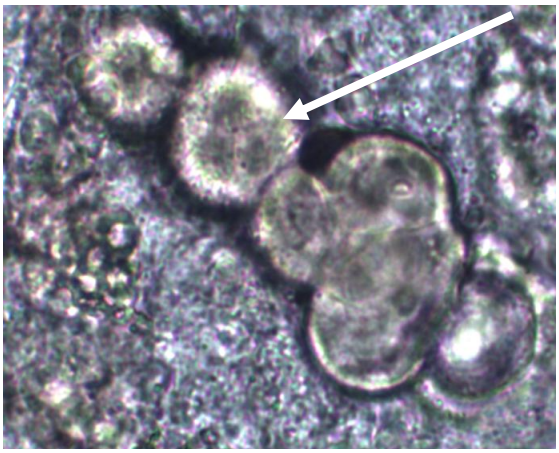

Figure 3.1: Wet mount of crystals in kidneys of birds consuming melamine.

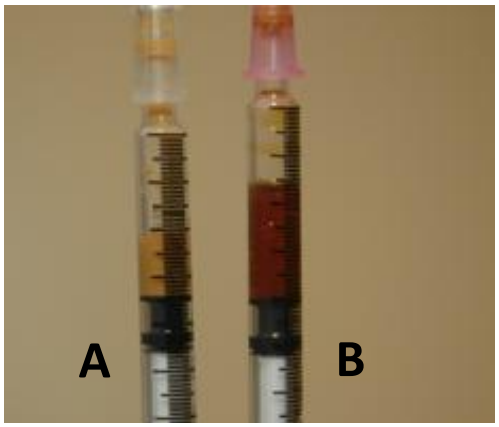

Figure 3.2: Photographs of bile; A) discolored, murky bile collected from a chicken fed 3% melamine and B) normal bile collected from a poult fed 0% melamine.

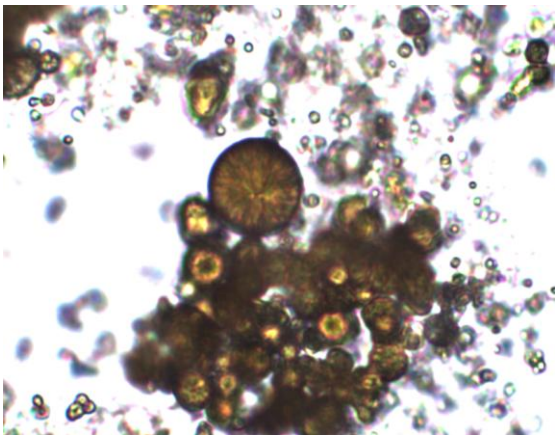

Figure 3.3: Wet mount of crystals observed in the bile of broilers fed melamine.

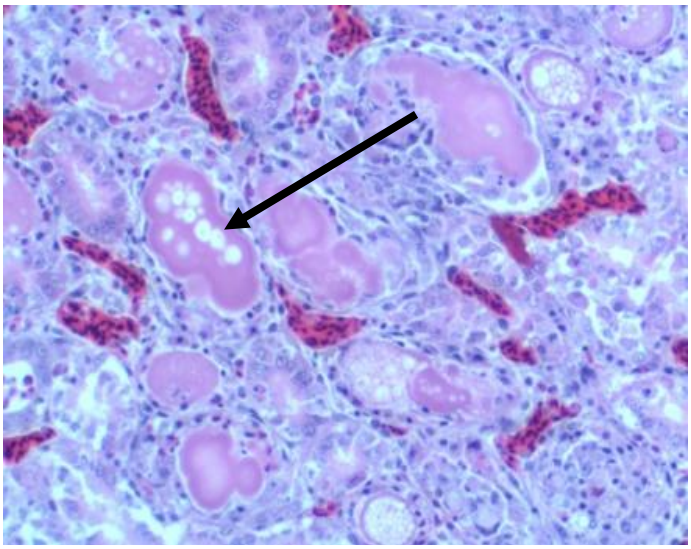

Figure 3.4: Kidney lesions of broilers fed melamine: mineralized casts in renal collecting tubules.

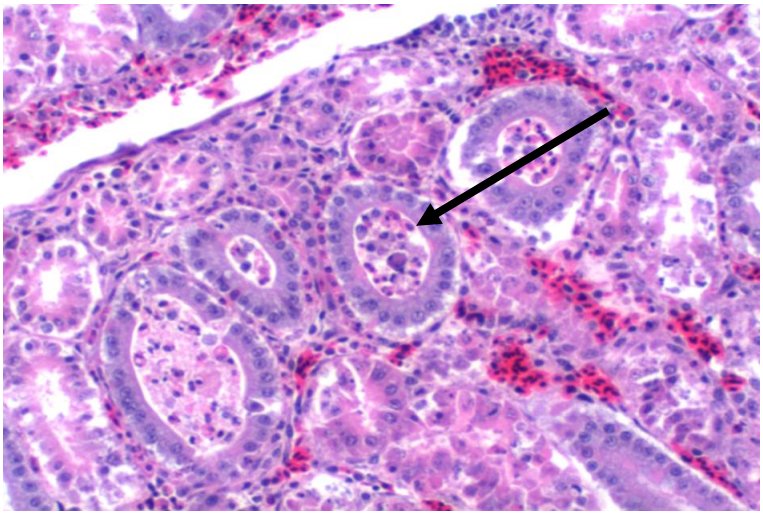

Figure 3.5: Kidney section revealing nephritis in broilers fed melamine.

CHAPTER 4

Effects of Cyanuric Acid in Young Broiler Chicks

Introduction

There have been recent concerns regarding adulteration of protein ingredients used in pet foods and human foods with melamine (M). Upon further investigation, it was determined that a combination of melamine and cyanuric acid (CA) was responsible for toxic effects in animals and humans (Brown et al., 2007). Cyanuric acid is a triazine (1,3,5-triazine-2,4,6-trihydroxy) with a molecular formula of $C_3H_3N_3O_3$ (OSHA, 2010). Cyanuric acid is the metabolite of the deaminated melamine compound. When the three NH_2 groups from melamine are replaced with an OH group, the resulting compound is cyanuric acid (Jutzi et al., 1982). Cyanuric acid, used in swimming pools as a stabilizer, is known to protect chlorine from degradation by the sun's ultraviolet rays (Cantú et al., 2001; Cyanuric).

On March 16th, 2007, Menu Foods placed a recall on wheat gluten and rice protein products imported from the Chinese food company, ChemNutra (Burns, 2007a; FDA 2007b). The contaminated products were recalled and instructed to be destroyed (FDA, 2007b). From April 5 to June 6, 2007, 235 cats (61% mortality) and 112 dogs (74%, mortality) were diagnosed with "pet food-induced nephrotoxicity" based on high concentrations of blood urea nitrogen and creatinine (Burns, 2007b). Studies, such as

those by Brown et al. (2007), found that melamine and cyanuric acid were present in renal tissue of affected animals. "Spoke-like" crystals, forming from consumed melamine and cyanuric acid, affected animal kidneys and were suspected to compromise kidney function, leading to acute renal failure and death in cats and dogs. Affected animals also developed lesions such as oral ulcers along the tongue and mineralization in the gastric mucosa, pulmonary smooth muscle, and alveolar walls (Brown et al., 2007; Cianciolo et al., 2008).

Furthermore, pigs and chickens consuming melamine and cyanuric acid contaminated pet food byproducts were traced to various states across the nation (Burns, 2007a; FDA, 2007a). Research by Filigenzi et al. (2007) determined that residues in the tissue of the affected livestock animals were not high enough to pose a threat to human health. Upon further investigation, it was determined that cyanuric acid alone can not be metabolized in mammals and therefore is generally excreted via urine (Dobson et al., 2008; Filigenzi et al., 2007).

The objective of this study was to determine if cyanuric acid alone will cause toxic effects in young chicks, and if so, at what dose will cyanuric acid become toxic.

Materials and Methods

Diet Preparation

Seven dietary treatments were prepared using graded levels of cyanuric acid purchased from Sigma-Aldrich Chemical Company (St. Louis, Mo.). Cyanuric acid was included at 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0% to a basal corn-soybean meal diet (Table

4.1) that was formulated to meet or exceed requirements of broiler chicks as recommended by the National Research Council (NRC, 1994). Cyanuric acid was substituted for sand to obtain the desired dietary cyanuric acid concentrations.

Birds, Management and Response Variables

One hundred and seventy five day-old male Ross broiler chicks were purchased from a commercial hatchery. Chicks were weighed, wing-banded, and sorted to a randomized block design in stainless steel battery pens. Housing was in an environmentally controlled room with a 24-hour constant light schedule. Feed and water were supplied *ad libitum*. Day-old chicks were divided among seven dietary treatments, five birds per pen, and five replicate pens per treatment and fed dietary treatments for 21 days. The animal care and use protocol was reviewed and approved by the University of Missouri Animal Care and Use Committee (ACUC).

On day 21, chicks were euthanized using carbon dioxide, weighed, and serum was collected from three birds per pen. Chicks that died before termination were weighed and sent to a pathology lab for necropsy. Residual feed was measured and recorded. At termination, kidneys, livers, and serum were collected from three birds per pen. Response variables included feed intake at 21 days, body weight gain, feed conversion (F:G), mortality, relative kidney and liver weights, gross histopathology and serum chemistry.

Statistical Analyses

Data for all response variables were subjected to regression analysis using the general linear models procedure of SAS software (SAS Institute, 2006). Regression

analysis best fit the means relative to a linear ($y_i = a + bx_i + E_i$), quadratic ($y_i = a + bx_i + cx^2_i + E_i$) or cubic ($y_i = a + bx_i + cx^2_i + dx^3_i E_i$) effect. Dunnett's test was run to calculate means among treatments and determine at which level of inclusion the specific parameter was significant from the control. Statistical significance was determined at a p-value less than or equal to 0.05.

Results

Mortality and Growth Performance

The effects of dietary treatments on mortality and growth performance are summarized in Table 4.2. Mortality was not different among broiler chicks fed cyanuric acid in levels up to 3.0% ($P > 0.05$). Feed intake and body weight gain did not show difference among treatments ($P > 0.05$). The ability of birds to convert feed to body weight gain (F:G) with increasing dietary concentrations of cyanuric acid also did not differ among treatments ($P > 0.05$).

Organ Weights

Effects of dietary treatment on relative kidney and liver weights are summarized in Table 4.3. Relative weights of kidney and liver were not affected ($P > 0.05$) by increasing dietary concentrations of cyanuric acid.

Serum Chemistry

Effects of dietary treatments on serum chemistries are summarized in Table 4.3. Birds fed up to 1.5% cyanuric acid did not show any significant differences in comparison to control values for serum glucose, albumin, total protein, calcium, aspartate

transaminase, gamma glutamyltransferase, and uric acid. However, a quadratic effect was observed in serum glucose values (P = 0.0053), a linear effect was observed in serum albumin values (P = 0.0354), and a cubic effect was observed in serum values for gamma glutamyltransferase (P = 0.0212).

Pathology

Gross Pathology - Early Mortality

The gross pathology of broilers fed 0, 2.0, 2.5, and 3.0% cyanuric acid that died early in the experiment were found to be unremarkable.

Histopathology and Bile Analysis - Early Mortality

Renal pathology was insignificant in all wet bile mounts of birds fed 0, 2.0, 2.5, and 3.0% cyanuric acid. No crystals were present in these bile samples.

Histopathology - Termination of Experiment

Histopathology of kidney sections among all treatments was unremarkable and revealed no renal pathology.

Discussion

In this study, mortality, feed intake, body weight gain, and feed conversion were not different among treatments (P > 0.05). These results suggest that cyanuric acid included in the diet at up to 3.0% is not toxic. These data support the study by Dobson et al. (2008) in which individual triazines fed to rats resulted in no observable toxicity. Significant mortality has not been reported in animals fed cyanuric acid alone (Reimschuessel et al., 2008). Only when cyanuric acid is combined with melamine, has

significant mortality, due to renal failure, been observed (Brown et al., 2007; Cianciolo et al., 2008; Jingbin et al., 2010; Puschner et al., 2007).

In the present study, relative kidney weights were similar among all treatments and were unaffected by addition of cyanuric acid, whereas animals fed combinations of melamine and cyanuric acid were found to have an increased relative kidney weight (Cianciolo et al., 2008; Dobson et al., 2008). In the study by Cianciolo et al. (2008), it was found that addition of cyanuric acid to a melamine contaminated diet altered the elimination kinetics of melamine, causing crystals to precipitate in the renal tubules, but otherwise individually fed triazines, such as cyanuric acid, did not precipitate in the kidney (Dobson et al., 2008; Reimschuessel et al., 2008). This finding is also supported by Reimschuessel et al. (2008) where melamine concentrations in fish tissue were higher than cyanuric acid concentrations when triazines were fed individually.

In contrast to the present study, the relative liver weights of birds fed melamine increased with increasing dietary levels of melamine. These results suggest that the cyanuric acid compound is able to be excreted without harm to the kidney or liver.

Examination of the wet mounts of bile in the early terminal birds found that cyanuric acid did not contribute to crystal formation. In contrast to our findings, Reimschuessel et al. (2008) observed pale gold crystals in trout fed cyanuric acid alone, which were distinguishable from the crystals formed by the melamine and cyanuric acid combination. Furthermore, histopathology and serum values were not altered in comparison to controls when cyanuric acid was included in ≤ 3.0% of the diet, and serum values fell within or near normal expected values for poultry species (Braun et al.,

1982; Coles, 1985 and 1997; and Puls, 1994). These data are supported by Reimschuessel et al. (2008) in which no significant serum or pathological effects were observed in pigs fed up to 0.4% cyanuric acid.

Results of this study indicate that cyanuric acid alone, at concentrations ≤ 3.0%, is not toxic to broilers fed dietary treatments from hatch to day 21.

Table 4.1. Ingredient and nutrient composition of basal ration

Item	Composition (%)
Ingredient	
Corn	55.45
Soybean Meal	30.25
Pork Meal	4.74
Corn Oil	4.21
Fish Meal	3.68
Dicalcium	0.36
Limestone	0.47
Salt	0.35
Methionine	0.19
Trace Mineral[1]	0.11
Vitamin Mix[2]	0.08
Selenium Mix[3]	0.06
Lysine	0.04
Copper Sulfate	0.00
Sand	3.00
Total	100
Nutrient composition (calculated)	
Crude Protein (%)	23.00
Metabolizable Energy (Kcal/kg)	3200
Lysine (%)	1.30
Methionine (%)	0.55
Methionine + Cysteine (%)	0.90
Threonine (%)	0.85
Calcium (%)	1.00
Phosphorus (% Av.)	0.45

[1]Trace mineral mix provided (mg/kg of diet): manganese, 110 mg from $MnSO_4$; iron, 60 mg from $FeSO_4 \bullet 7H_2O$; zinc, 110 mg from $ZnSO_4$; iodine, 2 mg from ethylenediamine dihydroidodide.
[2]Vitamin mix supplied (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.
[3]Selenium premix provided 0.2 mg of Se/kg of diet from $NaSeO_3$.

Table 4.2. Effects of cyanuric acid on performance of broiler chicks[1]

	Treatments[2] – Cyanuric acid %							ANOVA[5]		Regression[6]	
Item	0	0.5	1.0	1.5	2.0	2.5	3.0	Pooled SEM	P-value	L/Q	P-value
FI, g	904	893	915	958	913	886	884	21	0.2374	-	0.5060
BWG, g	763	730	754	760	759	730	731	17	0.5701	-	0.3330
F:G	1.208	1.224	1.213	1.262	1.223	1.234	1.250	0.016	0.2081	-	0.0771
Organ Weight[3]											
Kidney	0.93	0.91	0.93	0.85	0.96	0.94	0.90	0.43	0.1060	-	0.8865
Liver	3.10	2.98	2.93	2.87	3.03	3.08	2.93	0.19	0.7264	-	0.6987
Mort[4] %	8	0	0	0	4	8	12	4	0.2932	-	0.1683

[1]Data are means of 25 birds per treatment for growth performance parameters and 15 birds per treatment for organ weights.
[2]0 = Basal diet (BD); 0.5 = BD + 0.5% CA; 1.0 = BD + 1.0% CA; 1.5 = BD + 1.5% CA; 2.0 = BD + 2.0% CA; 2.5 = BD + 2.5% CA; 3.0 = BD + 3.0% CA.
[3]Relative organ weights expressed as a percentage of body weight.
[4]Mort = Mortality %
[5]One way analysis of variance values
[6]Regression- L = linear regression and Q = quadratic regression
*Signifies level at which cyanuric acid was toxic for specific parameters in comparison to control birds.

Table 4.3. Serum chemistries of broiler chicks fed graded levels of cyanuric acid[1]

Item[3]	Treatments[2] – Cyanuric acid %							ANOVA[4]		Regression[5]	
	0	0.5	1.0	1.5	2.0	2.5	3.0	Pooled SEM	P-value	L/Q/C	P-value
GLU (mg/dL)	289	248	255	212	244	269	267	23	0.0566	Q	0.0053
ALB (g/dL)	1.16	1.10	1.14	1.14	1.07	1.10	1.02	0.06	0.2911	L	0.0354
TP (g/dL)	2.61	2.61	2.68	2.78	2.62	2.62	2.54	0.12	0.5594	-	0.5706
GLOB (g/dL)	1.53	1.59	1.54	1.64	1.55	1.60	1.52	0.07	0.5752	-	1.0000
CA (mg/dL)	4.73	4.66	4.32	4.44	4.38	4.27	4.18	0.39	0.7810	-	0.1110
AST (IU/L)	202	228	223	217	208	201	155	36	0.5092	-	0.1269
GGT (U/L)	11.6	13.3	13.6	13.8	10.6	11.6	11.9	1.2	0.0788	C	0.0212
UA (mg/dL)	5.70	5.84	6.26	6.42	5.94	5.46	6.40	0.82	0.8725	-	0.7400

[1]Data are means of 15 birds per treatment.
[2]0 = Basal diet (BD); 0.5 = BD + 0.5% CA; 1.0 = BD + 1.0% CA; 1.5 = BD + 1.5% CA; 2.0 = BD + 2.0% CA; 2.5 = BD + 2.5% CA; 3.0 = BD + 3.0% CA.
[3]GLU = glucose, ALB = albumin, TP = total protein, GLOB = globulin, CA = calcium, AST = aspartate transaminase, GGT = gamma glutamyltransferase, UA = uric acid.
[4]One way analysis of variance values
[5]Regression- L = linear regression, Q = quadratic regression, and C = cubic regression

CHAPTER 5

Individual and Combined Effects of Melamine and Cyanuric Acid in Young Broiler Chicks

Introduction

Concerns regarding adulteration of protein ingredients used in pet foods and human foods with melamine (M) have resulted in the recall of food and feed products. Upon further investigation, it was determined that a combination of melamine and cyanuric acid (CA) was responsible for toxic effects in animals and humans (Brown et al., 2007). Melamine and cyanuric acid are triazine compounds that are normally used as chemicals in production of fire retardant materials and pool chlorine stabilizers, respectively (Lowy and Pritchard, 2008; Cantu et al., 2001). Melamine (1,3,5-triazine-2,4,6-triamine) can be degraded by removing three NH_2 groups and replacing them with OH groups to form cyanuric acid (1,3,5-triazine-2,4,6-trihydroxy; Jutzi et al., 1982).

On March 16th 2007, Menu Foods placed a recall on wheat gluten and rice protein products imported from the Chinese food company, ChemNutra (Burns, 2007a; FDA 2007b). The contaminated products were recalled and instructed to be destroyed (FDA, 2007b). From April 5 to June 6, 2007, 235 cats and 112 dogs (mortality was 61% and 74%, respectively) were diagnosed with "pet food-induced nephrotoxicity" based on high concentrations of blood urea nitrogen and creatinine (Burns, 2007b). Studies,

such as those by Brown et al. (2007), found that melamine and cyanuric acid were present in renal tissues of affected animals. "Spoke-like" crystals, formed from the consumption of melamine and cyanuric acid, affected animal kidneys and were suspected to compromise kidney function, leading to acute renal failure and death in cats and dogs. Affected animals also developed lesions such as oral ulcers along the tongue and mineralization in the gastric mucosa, pulmonary smooth muscle and alveolar walls (Brown et al., 2007; Cianciolo et al., 2008).

Furthermore, pigs and chickens consuming melamine and cyanuric acid contaminated pet food byproducts were traced to various states across the nation (Burns, 2007a; FDA, 2007a). Research by Filigenzi et al. (2007) determined that residues in the tissue of the affected livestock animals were not high enough to pose a threat to human health. Upon further investigation, it was determined that cyanuric acid alone can not be metabolized in mammals, and therefore is generally excreted via urine (Dobson et al., 2008; Filigenzi et al., 2007), unlike melamine, which has been found to accumulate in the renal tubules as crystals (Reimschuessel et al., 2008).

Combinations of melamine and its triazine derivatives such as cyanuric acid were studied in various animals. Puschner et al. (2007) found that cats fed combinations of high concentrations of melamine and cyanuric acid had severe kidney lesions and microscopic, green-brown circular crystals were found in the kidneys of cats fed a combination of 1% melamine and 1% cyanuric acid. Combinations of melamine and cyanuric acid in a 400/400 mg/kg/day mixture were considered toxic to rats (Jingbin et al., 2010). Likewise, toxicity was reported in rats fed melamine, ammeline, ammelide

and cyanuric acid in a 400/40/40/40 mg/kg/day mixture (Dobson et al., 2008). Research with fish fed melamine and cyanuric acid in combination resulted in white feces and excretion of nitrogenous waste via the gills of trout, salmon, and catfish, and death of two fish in the study (Reimschuessel et al., 2008). Reimschuessel et al. (2008) continued research in pigs and reported edema of the fascia around the kidneys of pigs administered dietary melamine and cyanuric acid in a 1:1 combination. Creatinine and blood urea nitrogen levels were elevated in affected swine, although values were not significantly different from control pigs.

The objective of this study was to determine the individual and combined effects of melamine and cyanuric acid in young chicks.

Materials and Methods

Diet Preparation

Ten dietary treatments were prepared using graded levels of melamine and cyanuric acid purchased from Sigma-Aldrich Chemical Company (St. Louis, Mo.). A control diet (basal diet) was formulated to meet or exceed requirements of broiler chicks as recommended by the National Research Council (NRC, 1994; Table 5.1). Three of the diets contained melamine alone at inclusion levels of 0.5, 1.0, and 1.5%. Another three diets contained cyanuric acid alone at inclusion levels of 0.5, 1.0, and 1.5%. The remaining three diets consisted of combinations of melamine and cyanuric acid in a 1:1 ratio totaling 1.0, 2.0, and 3.0% of the diet. Melamine and cyanuric acid were substituted for sand (up to 3.0%) in the basal diet.

Birds, Management and Response Variables

Two hundred and fifty day-old male Ross broiler chicks were purchased from a commercial hatchery. Chicks were weighed, wing-banded, and sorted to a randomized block design in stainless steel battery pens. Housing was in an environmentally controlled room with a 24-hour constant light schedule. Feed and water were supplied *ad libitum*. Day-old chicks were divided among 10 dietary treatments, five birds per pen, and five replicate pens per treatment, and fed dietary treatments for 21 days. The animal care and use protocol was reviewed and approved by the University of Missouri Animal Care and Use Committee (ACUC).

On day 21, chicks were euthanized using carbon dioxide and weighed. Chicks that died before termination were weighed and sent to a pathology lab for necropsy. Residual feed was measured and recorded. At termination, kidneys and livers were collected from three birds per pen to determine weights relative to final body weight. Serum samples were also collected via cardiac puncture from three birds per pen for determination of serum chemistries. In addition, samples of kidney were collected from five birds per treatment for histopathologic examination. Response variables included growth performance at 21 days (feed intake, body weight gain, and feed conversion), mortality, relative kidney and liver weights, serum chemistry, gross pathology, and histopathology.

Statistical Analyses

Data for all response variables were analyzed by the general linear model procedure of SAS software (SAS Institute, 2006) as a 3 x 3 factorial plus an additional

treatment. Individual means and interaction means were calculated for melamine and cyanuric acid treatments. Means for treatment showing significant differences in the main effect means were compared using Duncan's Multiple Range Test procedure. Statistical significance was determined at a p-value less than or equal to 0.05.

Results

Mortality and Growth Performance

The individual and combined effects of melamine and cyanuric acid on mortality and growth performance are summarized in Table 5.2. There was no effect ($P > 0.05$) of dietary treatments on mortality. Compared to controls, feed intake (FI) and body weight gain (BWG) were decreased ($P < 0.0001$) in birds fed melamine (FI, 762 vs 827 g; BWG, 592 vs 652 g) with the largest decrease observed in birds fed 1.5% melamine. No difference ($P > 0.05$) due to cyanuric acid inclusion was observed for feed intake or body weight gain. An interactive effect between melamine and cyanuric acid was significant for FI ($P = 0.0185$) and BWG ($P = 0.0035$). Compared with controls, birds fed the combination of melamine and cyanuric acid consumed on average 104 grams less feed and gained on average 85 grams less. The ability of birds to convert feed to body weight gain (F:G) with increasing dietary concentrations of melamine, cyanuric acid, or combinations of melamine and cyanuric acid did not differ among treatments ($P > 0.05$).

Organ Weights

Effects of dietary treatments on relative kidney and liver weights as a percent of body weight are summarized in Table 5.2. Compared to controls, chicks fed melamine

alone or the combination of melamine and cyanuric acid had heavier relative kidney weights (1.05 vs 0.90%) with the heaviest relative kidney weights observed in birds fed 1.5% melamine and in birds fed the highest combination of melamine and cyanuric acid. There was no effect ($P > 0.05$) of cyanuric acid on relative kidney weights. No interactive effect between melamine and cyanuric acid was observed for relative kidney weights ($P > 0.05$). Compared with controls, birds fed cyanuric acid had heavier relative liver weights (3.80 vs 3.51%). There was no effect ($P > 0.05$) of melamine alone on relative liver weights. An interactive effect between melamine and cyanuric acid was observed for relative liver weights ($P = 0.0345$). The interaction occurred because changes in relative liver weight differed in birds fed melamine alone, cyanuric alone, and the combination of melamine and cyanuric acid. Compared to controls which averaged 3.51%, relative liver weights of birds fed cyanuric acid alone and the combination of melamine and cyanuric acid averaged 3.80% and 3.92%, respectively.

Serum Chemistry

Effects of treatments on various serum values are presented in Table 5.3. A main effect for melamine alone and cyanuric acid alone was observed for serum aspartate transaminase (AST) levels ($P = 0.0420$ and $P = 0.0281$, respectively). Increasing levels of dietary melamine caused increased levels of AST, whereas increasing levels of dietary cyanuric acid caused a decrease in serum AST levels ($P < 0.05$). With the exception of AST, no individual or interactive effects between melamine and cyanuric acid were observed ($P > 0.05$) for any other serum values.

Pathology

The gross pathology of broilers that died early in the experiment and those that were euthanized at the end of the experiment were found to be unremarkable. Five kidneys were collected for histopathologic evaluation, however two samples from each combination treatment was lost during storage leaving only three kidney sections for histopathologic evaluation from these treatments. Kidney sections from control birds, birds fed melamine alone, and birds fed cyanuric alone were found to be unremarkable. Three of three kidney sections from birds fed the 0.5% combination diet had green spherical, spirally radiating crystals in the collecting tubules and collecting ducts (Figure 5.1). Two of three kidney sections from birds fed the 1.0% and 1.5% combination diets also had green spherical spirally radiating crystals in the collecting tubules and collecting ducts (Figure 5.1). Upon further observation, it was determined that these same crystals were polarizable (Figure 5.2).

Discussion

In this study, mortality was not significantly different among treatments, suggesting that melamine and cyanuric acid in combinations up to 3.0% (1.5% of each) will not cause mortality in broiler chicks fed dietary treatments from hatch to three weeks of age. These data are in contrast to previous research with cats, and dogs in which significant mortality associated with renal failure was observed (Brown et al., 2007; Cianciolo et al., 2008; Dobson et al., 2008). The lack of significance in mortality reflects previous research (Chapter 3), where mortality only became significant when

broilers were fed ≥ 2.5% melamine, and is comparable to Chapter 4 where ≤ 3.0% dietary cyanuric acid did not cause significant mortality in broilers.

Decreases in FI and BWG in birds fed melamine alone have been observed previously in broilers fed melamine alone (Chapter 3) but not in broilers fed cyanuric acid alone (Chapter 4). However, in those studies, depressions in FI and BWG were not observed until birds were fed 1.0% melamine. Lu et al. (2009) fed broilers up to 0.1% melamine for 42 days and observed no negative effects on growth performance. However, the highest level (0.1%) fed by Lu et al. (2009) was five-fold lower than the lowest level (0.5%) fed in the current study.

Decreased FI and BWG in broilers fed the combination of melamine and cyanuric acid appeared to be due primarily to melamine since cyanuric acid had no effect on either of these response variables. Interestingly, it appears that combinations containing higher concentrations of cyanuric acid may have ameliorated the effects of melamine since FI and BWG improved in birds fed 2.0% and 3.0% combinations compared to the 1.0% combination. Dobson et al. (2008) fed combinations of melamine and cyanuric acid (400 mg/kg/day) to rats and observed decreased FI and BWG. However, it is not possible from that study to determine whether the combination was more toxic to the rats since there were no dietary treatments that included melamine and cyanuric acid alone.

Increased relative kidney weight in birds fed melamine alone has been observed previously in broilers (Chapter 3) and ducks (Landers et al., 2010) fed melamine alone, and is consistent with previous reports indicating that the kidney is the target organ for

melamine. Increased relative kidney weights in broilers fed the combination of melamine and cyanuric acid appeared to be due primarily to melamine since cyanuric acid alone had no effect on relative kidney weight. Dobson et al. (2008) also observed increases in kidney weights in rats fed a combination of melamine and cyanuric acid.

In contrast to the kidney, increased liver weights relative to body weight in birds fed cyanuric acid alone or the combination of melamine and cyanuric acid would appear to be due primarily to cyanuric acid since melamine alone had no effect on relative liver weights. Data from the previous chapter (Chapter 3) suggests that broilers fed ≥ 2.5% melamine alone had increased relative liver weights, while relative liver weights of broilers fed ≤ 3.0% cyanuric acid alone (Chapter 4) were not different from relative liver weights of control birds.

With the exception of AST, individual and combined concentrations of melamine and cyanuric acid did not affect serum values. Moreover, all serum values, including serum AST values, fell within ranges reported as being normal for poultry species (Braun et al., 1982, Coles, 1985 and 1997, and Puls, 1994).

The crystals found in the kidneys of birds fed the combination diets have all of the characteristics of the polarizable melamine-cyanuric acid crystals described in the literature in cats, dogs, rats, and fish (Brown et al., 2007; Puschner et al., 2007; Cianciolo et al., 2008; Dobson et al., 2008; Reimschuessel et al. 2008). In those species, the formation of these insoluble crystals in the kidney results in kidney failure and eventual death. In the present study, however, the lesions caused by crystal formation were mild and were not severe enough to cause significant renal disease and mortality. Since the

combination levels of melamine and cyanuric acid fed in the current study were much higher than those fed in previous animal studies it would appear that broilers are able to excrete melamine and cyanuric acid more efficiently than cats and dogs. In a previous study (Chapter 3) it was observed that broilers excreted high concentrations of melamine in the bile. Biliary excretion has not been reported previously in mammals fed melamine and cyanuric acid which may not necessarily mean that there is no biliary excretion by mammals, but that biliary excretion has not been investigated in mammals. However Filigenzi et al. (2007) and Dobson et al. (2008) found that triazine compounds were not capable of being metabolized in mammals, therefore if there is no biliary excretion by mammals, this additional excretory route in the broiler could help explain the lower susceptibility of broilers to combinations of melamine and cyanuric acid observed in the present study. A second possible explanation for the reduced sensitivity of broilers to combinations of melamine and cyanuric acid may be the result of melamine-cyanuric acid crystal formation in the upper gastrointestinal tract of the bird, particularly the crop. When solutions of melamine and cyanuric acid are mixed together they have been shown to spontaneously form crystals (Reimschuessel et. al., 2008). In the present study, melamine-cyanuric acid crystals may have formed in the crop leading to increased fecal excretion of the two compounds and ultimately resulting in reduced absorption.

Results of the current study indicate that of the two triazines, melamine is more toxic than cyanuric acid. In addition, combinations of melamine and cyanuric acid resulted in the poorest growth performance compared to either triazine alone, and that

the combinations caused the formation of crystals in the kidney. In conclusion, the combination of melamine and cyanuric acid was more toxic to broilers than either triazine alone.

Table 5.1. Ingredient and nutrient composition of basal ration

Item	Composition (%)
Ingredient	
Corn	55.45
Soybean Meal	30.25
Pork Meal	4.74
Corn Oil	4.21
Fish Meal	3.68
Dicalcium	0.36
Limestone	0.47
Salt	0.35
Methionine	0.19
Trace Mineral[1]	0.11
Vitamin Mix[2]	0.08
Selenium Mix[3]	0.06
Lysine	0.04
Copper Sulfate	0.00
Sand	3.00
Total	100
Nutrient composition (calculated)	
Crude Protein (%)	23.00
Metabolizable Energy (Kcal/kg)	3200
Lysine (%)	1.30
Methionine (%)	0.55
Methionine + Cysteine (%)	0.90
Threonine (%)	0.85
Calcium (%)	1.00
Phosphorus (% Av.)	0.45

[1]Trace mineral mix provided (mg/kg of diet): manganese, 110 mg from $MnSO_4$; iron, 60 mg from $FeSO_4 \bullet 7H_2O$; zinc, 110 mg from $ZnSO_4$; iodine, 2 mg from ethylenediamine dihydroidodide.
[2]Vitamin mix supplied (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.
[3]Selenium premix provided 0.2 mg of Se/kg of diet from $NaSeO_3$.

Table 5.2. Effects of melamine and cyanuric acid on performance of broiler chicks[1]

Melamine (%)	Cyanuric Acid (%)	FI (g)	BWG (g)	F:G	Relative Kidney Weight[2]	Relative Liver Weight[2]	Mortality (%)
0	0	827	652	1.270	0.90	3.51	0
0.5	0	798	630	1.270	1.01	3.57	0
1.0	0	763	595	1.286	1.03	3.46	0
1.5	0	725	551	1.320	1.10	3.49	0
0	0.5	857	686	1.257	0.96	3.36	4
0	1.0	833	632	1.317	0.96	4.34	0
0	1.5	810	632	1.293	0.94	3.71	4
0.5	0.5	704	557	1.265	1.04	4.01	0
1.0	1.0	719	575	1.279	1.00	3.56	4
1.5	1.5	745	568	1.313	1.10	4.18	0
Pooled SEM[3]		21	16	0.030	0.05	0.38	2
Source of Variation		----------P----------					
Melamine (M)		<.0001	<.0001	0.6603	<.0001	0.1546	0.6122
Cyanuric Acid (CA)		0.1947	0.2330	0.7364	0.9013	0.0301	0.8410
M x CA		0.0185	0.0035	0.7160	0.5709	0.0345	0.2763
Main Effect Means							
Melamine	0	833[a]	650[a]	1.289	0.95[c]	3.80	3
	0.5	751[b]	593[b]	1.268	1.02[b]	3.79	0
	1.0	741[b]	585[b]	1.282	1.02[b]	3.51	2
	1.5	735[b]	560[b]	1.316	1.10[a]	3.84	0
Cyanuric Acid	0	762	592	1.292	1.05	3.51	0
	0.5	780	621	1.261	1.00	3.69	2
	1.0	776	603	1.298	0.98	3.95	2
	1.5	778	600	1.303	1.02	3.94	2

[1]Data are means of 25 birds per treatment for growth performance parameters and 15 birds per treatment for organ weights.
[2] Relative organ weights expressed as a percentage of body weight.
[3]One way analysis of variance values
[a-c]Means with different superscripts in a column differ significantly ($P < 0.05$).

Table 5.3. Serum chemistries of broiler chicks fed graded levels of melamine and cyanuric acid[1]

Melamine (%)	Cyanuric Acid (%)	GLU (mg/dL)	ALB (g/dL)	TP (g/dL)	GLOB (g/dL)	AST (IU/L)	GGT (U/L)	UA (mg/dL)
0	0	233	1.00	2.62	1.62	153	15.3	5.25
0.5	0	234	0.97	2.51	1.84	164	13.7	4.61
1.0	0	228	1.00	2.48	1.48	185	14.6	4.45
1.5	0	234	1.02	2.58	1.56	184	14.4	4.91
0	0.5	229	0.96	2.52	1.56	162	12.5	4.48
0	1.0	235	0.98	4.51	1.55	141	13.3	5.04
0	1.5	232	0.99	2.58	1.59	144	13.8	4.50
0.5	0.5	229	1.05	5.10	1.71	147	14.5	4.26
1.0	1.0	250	0.98	2.54	1.56	169	13.9	4.89
1.5	1.5	234	1.04	2.71	1.67	155	13.2	5.30
Pooled SEM[2]		14	0.04	1.36	0.15	14	1.0	0.68
Source of Variation		----------P----------						
Melamine (M)		0.9058	0.2412	0.6124	0.1125	0.0420	0.6353	0.6147
Cyanuric Acid (CA)		0.5566	0.6648	0.4882	0.8788	0.0281	0.9292	0.6447
M x CA		0.5953	0.2520	0.1943	0.6589	0.5843	0.1681	0.8046
Main Effect Means								
Melamine	0	232	0.98	3.20	1.57	149[c]	13.2	4.67
	0.5	232	1.01	3.81	1.78	156[bc]	14.1	4.44
	1.0	239	0.99	2.51	1.52	177[a]	14.3	4.67
	1.5	234	1.03	2.65	1.62	170[ab]	13.8	5.11
Cyanuric Acid	0	232	1.00	2.52	1.63	178[a]	14.2	4.66
	0.5	229	1.01	3.81	1.64	155[b]	13.5	4.37
	1.0	243	0.98	3.53	1.56	155[b]	13.6	4.97
	1.5	233	1.02	2.65	1.63	150[b]	13.5	4.90

[1]Data are means of 15 birds per treatment: GLU = glucose, ALB = albumin, TP = total protein, GLOB = globulin, CA = calcium, AST = aspartate transaminase, GGT = gamma glutamyltransferase, UA = uric acid.
[2]One way analysis of variance values
[a-c]Means with different superscripts in a column differ significantly ($P < 0.05$).

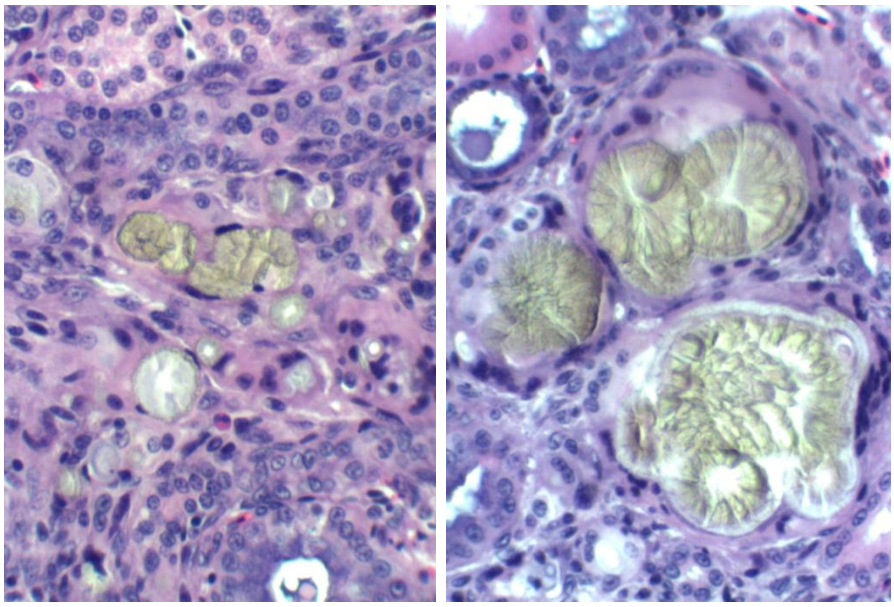

Figure 5.1: Kidney lesions observed in broilers fed combinations of melamine and cyanuric acid. Crystals observed under normal light.

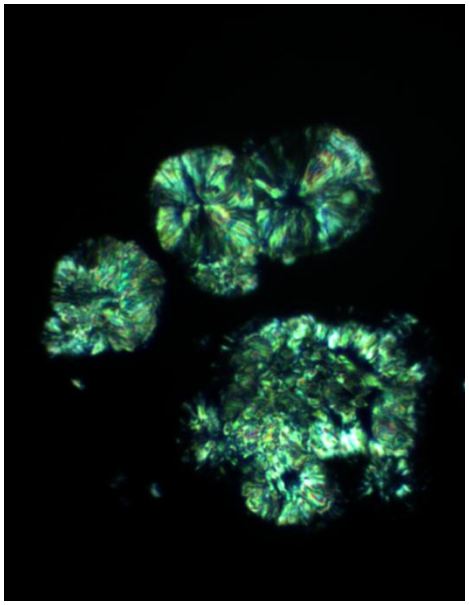

Figure 5.2: Kidney lesions observed in broilers fed a combination of melamine and cyanuric acid. Crystals observed under polarized light.

CHAPTER 6

Effects of Melamine in Young Turkey Poults

Introduction

Concerns regarding adulteration of protein ingredients used in pet foods and human foods with melamine (M) have been of current interest. Melamine (1, 3, 5-triazine-2, 4, 6-triamine) is a white, crystalline compound with a molecular formula of $C_3H_6N_6$ (NIOSH, 2006; OSHA, 2009; Safety Data, 2008) and is typically used in industry as a synthetic chemical for production of resins, foams, cleaning products, fertilizer (although prohibited from use in the USA as a fertilizer), pesticides, and fire retardant materials (FDA, 2007a; Lowy and Pritchard, 2008). More recently, melamine has been detected in ingredients fed to animals and humans. The Kjeldahl and Dumas methods are used in nutrition to determine the amount of protein in a given ingredient or diet based off of the amount of nitrogen in the sources. The amount of nitrogen in the source is then multiplied by 6.25 to determine protein content. Due to melamine's chemical structure containing 66% nitrogen by mass, the Kjeldahl or Dumas method indicate melamine to be rich in protein, implying that this product would be useful in increasing protein in a diet. Unfortunately, these methods allow room for error in determining true protein availability, especially when used to evaluate products that

contain compounds such as melamine, which are nitrogen rich, yet nutritionally inadequate (Ingelfinger, 2008).

On March 16th 2007, it was announced by Menu Foods that a voluntary recall was placed on wheat gluten and rice protein products imported from the Chinese food company, ChemNutra (Burns, 2007a; FDA 2007b). Ingredients contaminated with melamine including pet food, fish feed, and vegetable proteins were instructed to be recalled and destroyed (FDA, 2007b). The Food and Drug Administration (2007c) listed over 100 brands involved in the pet food recall, making it one of the largest pet food safety problem in the United States (Turley, 2009). From April 5 to June 6, 2007, 235 cats (61% mortality) and 112 dogs (74% mortality) were diagnosed with "pet food-induced nephrotoxicity" based on high concentrations of blood urea nitrogen and creatinine (Burns, 2007b). Lesions and deposits of "spoke-like" melamine crystals in the renal tubules were indicated as the cause of renal failure in chickens fed greater than or equal to 1.0% melamine (Chapter 3), and had also been reported to cause death in affected cats and dogs (Brown et al., 2007). Some animals developed lesions including oral ulcers along the tongue and mineralization in the gastric mucosa, pulmonary smooth muscle, and alveolar walls (Brown et al., 2007; Cianciolo et al., 2008).

Furthermore, by-products of the melamine contaminated ingredients were traced across the United States and had been consumed by pigs and chickens (Burns, 2007a; FDA, 2007a). Filigenzi et al. (2007) determined that melamine residues in the tissues of affected animals were not high enough to pose a threat to human health. Distribution of melamine may be largely limited to the extracellular fluid, therefore

reducing absorption into most organs (Baynes et al., 2008). This is further supported by research (Chapter 3) in which chickens fed high concentrations of melamine (5,000 ppm) had severely diluted melamine residues in the kidney (151 ppm) and liver (257 ppm). Upon further investigation, it was determined that melamine and its related triazine compounds are generally excreted via the kidneys and are not metabolized in mammals (Dobson et al., 2008; Filigenzi et al., 2007).

In order to better understand the effects of melamine in avian species, this chapter is presented as a follow up to the previously presented research with broiler chicks (Chapter 3). The objective of this current study was to determine if melamine will cause toxic effects in turkey poults, as was observed in chicks, and if so, at what dose of melamine will toxicity occur.

Materials and Methods

Diet Preparation

Seven dietary treatments were prepared using graded levels of melamine purchased from Sigma-Aldrich Chemical Company (St. Louis, Mo.). Melamine was included at 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0% to a basal corn-soybean meal diet (Table 6.1) that was formulated to meet or exceed requirements of turkey poults as recommended by the National Research Council (NRC, 1994). Melamine was substituted for sand to obtain the desired dietary melamine concentrations.

Birds, Management and Response Variables

One hundred and seventy five day-old male Nicholas turkey poults were purchased from a commercial hatchery in California, Missouri. Poults were weighed, wing-banded, and sorted to a randomized block design in stainless steel battery pens. Housing was in an environmentally controlled room with a 24-hour constant light schedule. Feed and water were supplied for *ad libitum* consumption. Day-old poults were divided among seven dietary treatments (five birds per pen and five replicate pens per treatment) and fed dietary treatments for 21 days. The animal care and use protocol was reviewed and approved by the University of Missouri Animal Care and Use Committee (ACUC).

On day 21, poults fed ≤ 1.5% melamine were euthanized using carbon dioxide, weighed, and serum was collected via cardiac puncture from three birds per pen. Poults that died before termination were weighed and sent to a pathology lab for necropsy. Due to significant mortality, poults fed ≥ 2.0% melamine were terminated at day 15. Residual feed was measured and recorded. At termination, serum, kidney, liver, bile, and breast muscle samples were collected from three birds per pen. Tissue samples were frozen for further analysis. Response variables included feed intake, body weight gain, feed conversion (F:G), mortality, serum chemistry, relative kidney and liver weights, gross and histopathology of kidneys, and melamine concentrations in breast muscle, kidney, liver, and bile.

Melamine Analysis

Melamine residue levels were measured in kidney and breast muscle using high-performance liquid chromatography (HPLC) analysis. An 87:13 buffer to acetonitrile (of

HPLC grade) mobile phase was used to extract melamine from samples. The buffer was 10 mM citric acid plus 10 mM sodium octanesulfate (pH 3) into one liter of distilled water. The pH was adjusted with addition of NaOH 1 N until the solution reached a pH of 3. The calibration curve used standards of 1, 2, 5, and 10 ppm. A Phenomenex Luna 5 µC18 250 X 4.60 mm column was used for analysis.

A series of concentrations of standard melamine solutions were prepared by dilution of the stock solution. Addition of 9.9 mL of a 1:1 buffer:ACN solution to 0.1 mL original 1000 ppm standard to obtain 10 ppm, 0.5 mL of the buffer:ACN solution with the addition of 0.5 mL of 10 ppm standard to obtain 5 ppm, and 0.9 mL of 1:1 buffer:ACN added to 0.1 mL of 10 ppm standard to obtain 1 ppm.

For tissue analysis, 0.25 g of tissue was added to 2 mL of a 1:2 water/ACN solution. Samples were ground and put into Falcon tubes™. The primary tubes were rinsed two times with the 1:2 water/ACN solution. Samples were sonicated for 30 minutes each and then vortexed for one minute. Samples were divided among two tubes and centrifuged for 5 minutes (32,000 rpm). Liquid was filtered from the sample through an M224 column. A 750 µL of sample was added to 250 µL of buffer. The samples were then analyzed by HPLC using UV detection.

Statistical Analyses

Data for all response variables were subjected to regression analysis using the general linear models procedure of SAS (SAS Institute, 2006). Regression analysis best fit the means relative to a linear ($y_i = a + bx_i + E_i$) and/or quadratic ($y_i = a + bx_i + cx^2_i + E_i$) response. Dunnett's test was run to calculate means among treatments and determine

at which level of inclusion the specific parameter was significant from the control. Statistical significance was accepted at a p-value of ≤ 0.05.

Results

Mortality and Growth Performance

The effects of dietary treatments on mortality and growth performance are summarized in Table 6.2. Thirty seven of the 44 treatment related deaths occurred by day 10 of the experiment. Due to 63, 93, and 93% mortality in birds fed 2.0, 2.5, and 3.0% melamine, respectively, birds remaining in the study at day 15 were terminated and growth performance data were not available for these birds. Mortality increased linearly ($P = 0.0056$) with increasing dietary concentrations of melamine in poults fed up to 1.5% melamine. Feed intake ($P = 0.0248$) and body weight gain ($P = 0.0018$) decreased linearly with increasing dietary concentrations of melamine up to 1.5%. Feed efficiency also worsened linearly with increasing levels of dietary melamine up to 1.5%.

Organ Weights

Effects of dietary treatments on relative kidney and liver weights are summarized in Table 6.2. Relative kidney weights and relative liver weights increased linearly ($P < 0.0001$ and $P = 0.0473$, respectively) with increasing dietary concentrations of melamine.

Serum Chemistry

Effects of dietary treatments on serum chemistries are summarized in Table 6.3. Birds fed up to 1.5% melamine did not show any significant differences in comparison to

control values for serum glucose, albumin, total protein, calcium, aspartate transaminase, or uric acid.

Tissue Residues

Table 6.4 contains a summary of the effects of dietary treatments on melamine residues in breast muscle and kidney. Melamine residues in kidney tissue increased linearly ($P < 0.0001$) with increasing dietary concentrations of melamine, whereas melamine residues in breast muscle increased quadratically ($P < 0.0105$) with increasing dietary concentrations of melamine. Compared with controls, melamine concentrations in breast muscle were higher in birds fed ≥ 1.0%, whereas melamine concentrations in kidney tissue were higher ($P < 0.0001$) in birds fed all levels of melamine. Melamine residues, averaged across all dietary treatments, were highest in the kidney (311 ppm) and lowest in breast muscle (212 ppm).

Pathology

Gross Pathology - Early Mortality

No gross lesions associated with melamine toxicity were observed in early mortality poults fed 0 or 0.5% melamine. At necropsy it was observed that one of three poults fed 1.0% melamine, five of seven poults fed 1.5% melamine, eight of eight poults fed 2.0% melamine, 14 of 15 poults fed 2.5% melamine, and 17 of 17 poults fed 3.0% melamine exhibited gross pathology characteristics associated with renal failure due to melamine toxicity. Gross pathology of birds indicating melamine toxicity include pale, enlarged kidneys, urate crystals on the liver and/or pericardium, white crystals visible by eye in bile, and/or renal hemorrhage.

Histopathology - Early Mortality

Birds fed 1.0, 1.5, 2.0, 2.5, and 3.0% melamine were found to have significant renal histopathology. One kidney section of a bird fed 1.0% melamine revealed eosinophilic to basophilic mineralized casts in the collecting tubules and collecting ducts. Likewise, similar casts were noted in five, eight, 14, and 17 kidney sections of early mortality birds fed 1.5, 2.0, 2.5, and 3.0% melamine, respectively. Kidney sections of these birds often revealed mild to severe heterophil infiltration in the medullary cone, and mineralized casts within the distal and proximal convoluted tubules, collecting tubules, and collecting ducts. Microscopic crystals < 2 microns in bile were also observed. These lesions are associated with melamine toxicity.

Gross Pathology - Termination of Experiment

High mortality in treatments fed ≥ 2.0% melamine resulted in early termination at 15 days of age. For all other treatments where birds survived to 21 days of age, gross examination revealed differences among treatments which are best characterized by body weight data (Figure 6.1). Gross examinations of the poults were otherwise unremarkable and all tissues, including the liver and kidney, appeared normal.

Histopathology - Termination of Experiment

Histopathology of the liver from all treatments and the kidney of birds fed 0 and 0.5% melamine were unremarkable. In birds fed 1.0% melamine, one kidney section had isolated foci of mild interstitial heterophil infiltration, whereas four other kidney sections were unremarkable. Two of five kidney sections of birds fed 1.5% melamine

were unremarkable, while two sections revealed mild interstitial heterophil infiltration and one section revealed moderate interstitial heterophil infiltration with isolated mineralized casts within the collecting tubules. Two of five kidney sections of birds fed 2.0% melamine were found to have mild interstitial heterophil infiltration associated with isolated mineralized casts noted within the collecting tubules. Two kidney sections of birds fed 2.5% melamine revealed mild interstitial heterophil infiltration associated with isolated mineralized casts within the collecting tubules while another two kidney sections of birds fed 2.5% melamine revealed mild multifocal interstitial heterophil infiltration (Figure 6.2). One of three kidney specimens available for examination from birds fed 3.0% melamine revealed mild interstitial heterophil infiltration with isolated mineralized casts noted within the collecting tubules. The overall conclusion was that the lesions (Figure 6.2) noted were generally mild and would not significantly affect renal function. Although melamine toxicity caused significant mortality due to renal failure during the experiment, it was found that poults surviving to either 14 days (birds fed 2.0, 2.5, and 3.0% melamine) or those surviving to day 21 (birds fed 0, 0.5, 1.0, and 1.5% melamine) had minimal histopathologic changes in the kidney.

Discussion

In this study, mortality was ≥ 32% in birds fed ≥ 1.5% melamine, however 84% of this mortality occurred by day 10. Due to high mortality, remaining birds fed ≥ 2.0% melamine were terminated and results other than residue levels are unavailable for

analysis. In the only other poultry study found in the literature (Lu et al., 2009) in which melamine was fed to broilers, concentrations of melamine up to 0.1% of the diet did not cause mortality. However, significant mortality has been reported previously in cats and dogs consuming food contaminated with both melamine and cyanuric acid, and the mortality was attributed to renal failure in these species (Brown et al., 2007; Cianciolo et al., 2008). Renal failure was considered to be the cause of the mortality in the present study in which poults were fed only melamine, and was also the diagnosis in rats fed combinations of melamine and cyanuric acid (Dobson et al., 2008). These data indicate, at least in poults, that high dietary concentrations of melamine alone will cause mortality due to renal failure.

In the present study, growth performance of poults was not negatively affected until birds were fed ≥ 1.0% melamine, with feed intake decreased by 14% and body weight gain depressed by 18% compared to controls. These results are consistent with a previous study by Lu et al. (2009) in which growth performance was not negatively affected in broilers fed up to 0.1% melamine for 42 days. Decreased growth and feed intake have been reported in cats and rats consuming melamine/cyanuric acid contaminated food (Dobson et al., 2008; Cianciolo et al., 2008).

Increased uric acid levels reported in rats (Jingbin et al., 2010), cats, and dogs (Brown et al., 2007; Cianciolo et al., 2008; Dobson et al., 2008) fed melamine and cyanuric acid was not observed in the present study in birds fed up to 1.5% melamine. In fact, no serum values were reported to be outside of normal ranges, suggesting that melamine is effectively filtered out in the kidney despite these animals developing renal

failure. This may be supported by the idea that wastes are cleared from the body more effectively due to biliary excretion in birds fed high concentrations of melamine.

Increases in relative kidney (≥ 1.0% melamine) weights observed in the present study have been observed previously in cats (Cianciolo et al., 2008) and rats (Dobson et al., 2008) fed food containing melamine and cyanuric acid, and are consistent with the kidney being the target organ of melamine/cyanuric acid. In contrast to Chapter 3, where melamine ≥ 2.5% caused increases in relative liver weights in broilers, the current study found no significant change in relative liver weights of poults fed melamine alone.

Tissue analysis indicate that melamine residues were highest in the kidney (311 ppm) and lowest in breast muscle (212 ppm). Compared to breast muscle, higher melamine concentrations were also observed in a previous study (Chapter 3) in which broilers were fed up to 3.0% melamine. These results are also consistent with a previous report by Lü et al. (2009) who also observed a similar tissue distribution in broilers fed melamine in concentrations ranging from 0 to 0.1%. High melamine concentrations in the kidney are also consistent with previous reports indicating that the kidney is the primary organ for melamine toxicity where melamine crystals are known to form and block collecting tubules (Brown et al., 2007; Cianciolo et al., 2008; Dobson et al., 2008; Ingelfinger, 2008).

Gross and histopathology findings are compatible with the lesions of melamine toxicity that were previously reported in broiler chickens (Chapter 3). Moderate to severe tubulointerstitial nephritis with mineralized casts within the collecting tubules and collecting ducts were noted in poults and are consistent with previous findings in

cats and dogs (Brown et al., 2007 and Cianciolo et al., 2008; Dobson et al., 2008). The kidney histological results in the present study were similar to histology results of rats fed a combination of 400 mg/kg/day of dietary melamine and 400 mg/kg/day of cyanuric acid (Dobson et al., 2008) and cats, dogs, and fish fed feed containing both melamine and cyanuric acid (Brown et al., 2007; Cianciolo et al., 2008; Reimschuessel et al., 2008). Mortality observed in studies with cats, dogs, and rats was attributed to deposits of melamine/cyanuric acid crystals that block renal tubules leading to renal failure. Deposits of melamine crystals appear to be responsible for the renal failure and mortality observed in the present study, in which all affected animals were reported to have pale, enlarged kidneys.

The overall conclusion in relation to lesions was that poults surviving to day 14 (birds fed 2.0, 2.5, and 3.0% melamine) or those surviving to day 21 (birds fed 0, 0.5, 1.0, and 1.5% melamine) were less sensitive to melamine or that surviving birds were able to more efficiently excrete melamine. Lu et al. (2009) reported that tissue melamine residues were lower in birds fed melamine for 42 days compared to those fed melamine for 28 days and hypothesized that broilers may have developed more capacity to clear melamine from body tissues with advancing age.

Results of this study indicate that like broilers, melamine at concentrations less than or equal to 1.5% is toxic to poults fed dietary treatments from hatch to day 21. Similarly, poults can tolerate up to 5,000 mg melamine/kg diet with no adverse effects on growth performance but at this dietary concentration kidneys contain significant concentrations of melamine.

Table 6.1. Ingredient and nutrient composition of basal ration

Item	Composition (%)
Ingredient	
Soybean Meal	50.60
Corn	39.23
Corn Oil	2.77
Dicalcium	2.42
Limestone	1.14
Salt	0.39
Methionine	0.21
Trace Mineral[1]	0.10
Selenium Mix[2]	0.08
Vitamin Mix[3]	0.06
Copper Sulfate	0.004
Sand	3.00
Total	100
Nutrient composition (calculated)	
Crude Protein (%)	28.00
Metabolizable Energy (Kcal/kg)	2800
Lysine (%)	1.60
Methionine (%)	0.62
Methionine + Cysteine (%)	1.05
Threonine (%)	1.06
Calcium (%)	1.20
Phosphorus (% Av.)	0.60

[1]Trace mineral mix provided (mg/kg of diet): manganese, 110 mg from $MnSO_4$; iron, 60 mg from $FeSO_4 \bullet 7H_2O$; zinc, 110 mg from $ZnSO_4$; iodine, 2 mg from ethylenediamine dihydroidodide.
[2]Selenium premix provided 0.2 mg of Se/kg of diet from $NaSeO_3$.
[3]Vitamin mix supplied (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 6.2. Effects of melamine on performance of turkey poults[1]

	Treatments[2] – Melamine %				ANOVA[5]		Regression[6]	
Item	0	0.5	1.0	1.5	Pooled SEM	P-value	L/Q	P-value
FI, g	826	801	707	660	51	0.1346	L	0.0248
BWG, g	581	598	475	434*	33	0.0081	L	0.0018
F:G	1.422	1.350	1.607	1.750	0.111	0.0897	L	0.0251
Organ Weight[3]								
Kidney	1.09	1.15	1.32*	1.36	0.06	<.0001	L	<.0001
Liver	2.64	2.59	2.76	2.82	0.13	0.1530	L	0.0473
Mort[4] %	0	4	12	32*	7	0.0298	L	0.0056

[1]Data are means of 25 birds per treatment.
[2]0 = Basal diet (BD); 0.5 = BD + 0.5% M; 1.0 = BD + 1.0% M; 1.5 = BD + 1.5% M.
[3]Relative organ weights expressed as a percentage of body weight.
[4]Mort = Mortality %
[5]One way analysis of variance values
[6]Regression- L = linear regression and Q = quadratic regression
*Signifies level at which melamine became toxic for specific parameters in comparison to control birds.

Table 6.3. Serum chemistries of turkey poults fed graded levels of melamine[1]

	Treatments[2] – Melamine %				ANOVA[4]		Regression[5]	
Item[3]	0	0.5	1.0	1.5	Pooled SEM	P-value	L/Q	P-value
GLU (mg/dL)	331	351	338	345	29	0.8456	-	0.7009
ALB (g/dL)	0.76	0.95	0.89	0.80	0.12	0.2183	-	0.8839
TP (g/dL)	2.30	2.67	2.60	2.47	0.23	0.2379	-	0.5116
CA (mg/dL)	5.49	6.05	6.05	6.16	0.46	0.3074	-	0.1091
AST (IU/L)	218	232	214	220	18	0.6661	-	0.8116
UA (mg/dL)	8.06	7.01	6.95	7.79	1.37	0.7121	-	0.8445

[1]Data are means of 15 birds per treatment.
[2]0 = Basal diet (BD); 0.5 = BD + 0.5% M; 1.0 = BD + 1.0% M; 1.5 = BD + 1.5% M.
[3]GLU = glucose, ALB = albumin, TP = total protein, CA = calcium, AST = aspartate transaminase, UA = uric acid.
[4]One way analysis of variance values
[5]Regression- L = linear regression and Q = quadratic regression
*Signifies level at which melamine became toxic for specific parameters in comparison to control birds.

Table 6.4. Residue levels of melamine in breast muscle and kidney of turkey poults[1]

	Treatments[2] – Melamine %							ANOVA[3]		Regression[4]	
Item	0	0.5	1.0	1.5	2.0	2.5	3.0	Pooled SEM	P-value	L/Q	P-value
Breast muscle	15	63	249*	298	447	451	926	14	<.0001	Q	0.0105
Kidney	10	165*	370	398	764	586	1071	31	<.0001	L	<.0001

[1] Melamine residual levels in breast muscle (6 samples per treatment for ≤ 1.5% melamine and one sample per treatment for ≥ 2.0% melamine) and kidney (6 samples per treatment for ≤ 1.5% melamine and one sample per treatment for ≥ 2.0% melamine) melamine residual levels measured in ppm.

[2]0 = Basal diet (BD); 0.5 = BD + 0.5% M; 1.0 = BD + 1.0% M; 1.5 = BD + 1.5% M; 2.0 = BD + 2.0% M; 2.5 = BD + 2.5% M; 3.0 = BD + 3.0% M.

[3]One way analysis of variance values

[4]Regression- L = linear regression and Q = quadratic regression

*Signifies level at which melamine residue levels became significant in comparison to control.

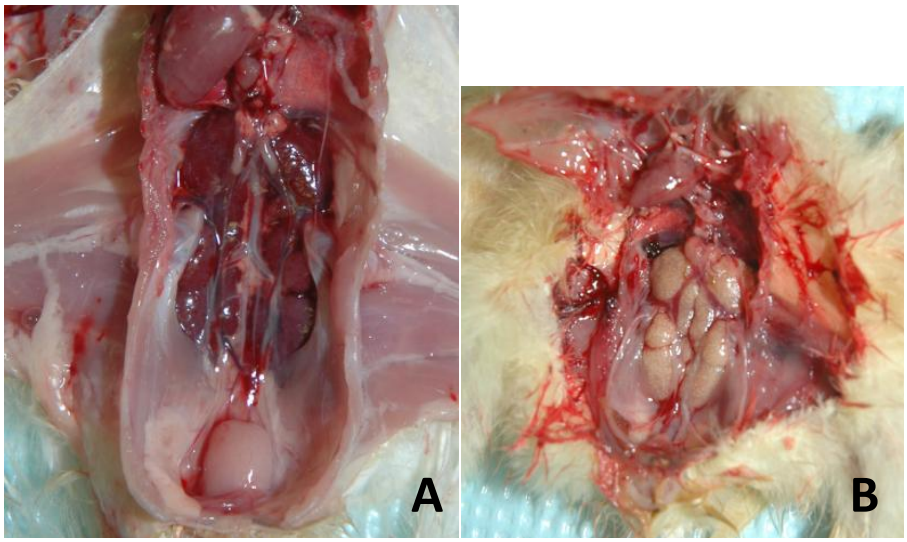

Figure 6.1: Gross pathology of turkeys: A) normal kidneys of a control bird fed 0% melamine and B) pale and enlarged kidneys of a bird fed 2% melamine.

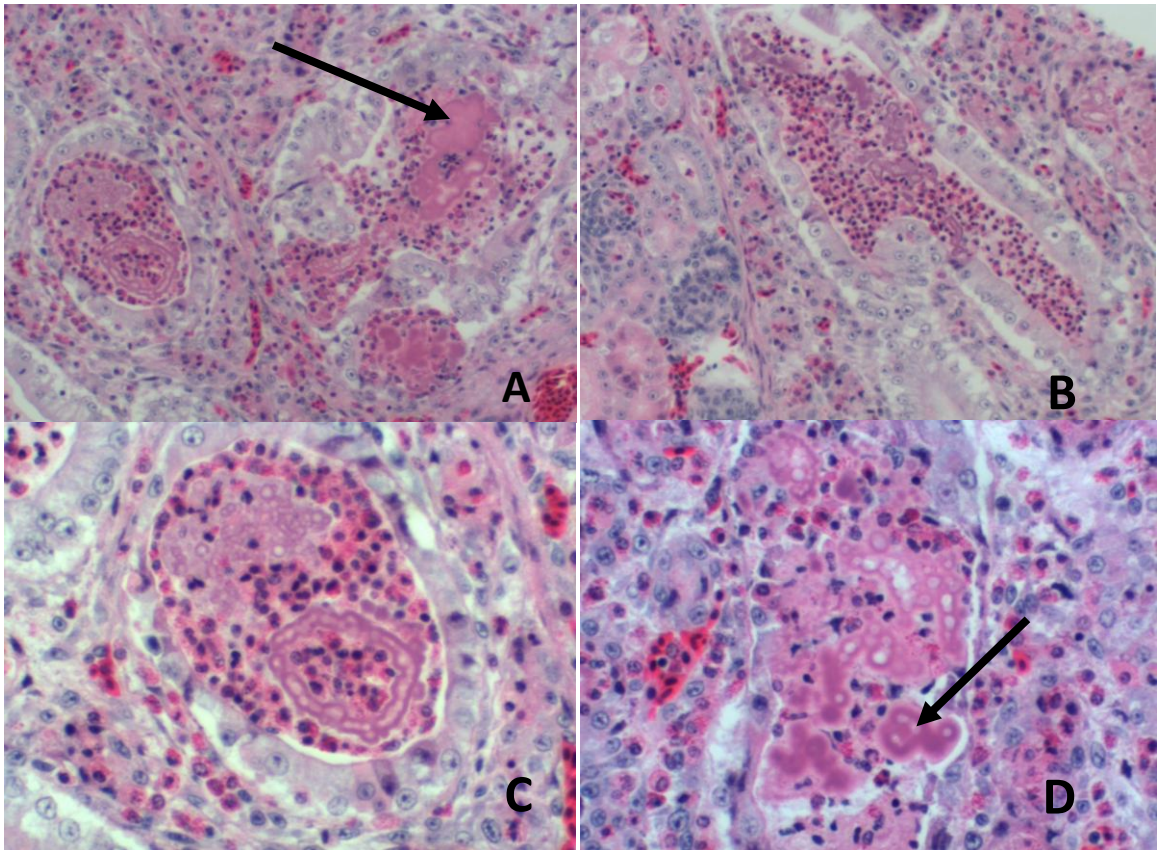

Figure 6.2: Photomicrographs of kidneys of poults fed melamine; A and B) 100x magnification; C and D) 200x magnification. Arrows indicate melamine crystals.

CHAPTER 7

Effects of Cyanuric Acid in Young Turkey Poults

Introduction

Concerns regarding adulteration of protein ingredients, used in pet foods and human foods, with melamine (M) have led to recent investigations in cyanuric acid (CA), the deaminated derivative of the melamine compound. Cyanuric acid is a triazine (1,3,5-triazine-2,4,6-trihydroxy) with a molecular formula of $C_3H_3N_3O_3$ (OSHA, 2010). When all three NH_2 groups from melamine are replaced with an OH group, the resulting compound is cyanuric acid (Jutzi et al., 1982). Cyanuric acid, used in swimming pools as a stabilizer, is known to protect chlorine from degradation by the sun's ultraviolet rays (Cantú et al., 2001; Cyanuric). The combination of melamine and cyanuric acid has been known to cause toxic effects in animals and humans (Brown et al., 2007).

On March 16th 2007 it was announced by Menu Foods that a voluntary recall was placed on wheat gluten and rice protein products imported from the Chinese food company, ChemNutra (Burns, 2007a; FDA 2007b). Ingredients contaminated with melamine and/or cyanuric acid, including pet food, fish feed and vegetable proteins, were instructed to be recalled and destroyed (FDA, 2007b). From April 5 to June 6, 2007, 235 cats (61% mortality) and 112 dogs (74% mortality) were diagnosed with "pet food-induced nephrotoxicity" based on high concentrations of blood urea nitrogen and

creatinine (Burns, 2007b). Studies, such as those by Brown et al. (2007), found that melamine and cyanuric acid were present in renal tissue of affected animals. "Spoke-like" crystals, forming from consumed melamine and cyanuric acid, affected animal kidneys and were suspected to compromise kidney function, leading to acute renal failure and death in cats and dogs. Affected animals also developed lesions such as oral ulcers along the tongue and mineralization in the gastric mucosa, pulmonary smooth muscle, and alveolar walls (Brown et al., 2007; Cianciolo et al., 2008).

Furthermore, pigs and chickens consuming melamine and cyanuric acid contaminated pet food byproducts were traced to various states across the nation (Burns, 2007a; FDA, 2007a). Research by Filigenzi et al. (2007) determined that residues in the tissue of the affected livestock animals were not high enough to pose a threat to human health. Upon further investigation, it was determined that cyanuric acid alone can not be metabolized in mammals, and therefore is generally excreted via urine (Dobson et al., 2008; Filigenzi et al., 2007).

In order to better understand the effects of cyanuric acid in avian species, this chapter is presented as a follow up to the previously presented research with broiler chicks (Chapter 4). The objective of this current study was to determine if turkey poults fed a cyanuric acid contaminated diet would exhibit toxic effects, and if so, at what dose would toxicity occur.

Materials and Methods

Diet Preparation

Seven dietary treatments were prepared using graded levels of cyanuric acid purchased from Sigma-Aldrich Chemical Company (St. Louis, Mo.). Cyanuric acid was included at 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0% to a basal corn-soybean meal diet (Table 7.1) that was formulated to meet or exceed requirements of turkey poults as recommended by the National Research Council (NRC, 1994). Cyanuric acid was substituted for sand (up to 3.0%) in the diet.

Birds, Management and Response Variables

One hundred and seventy five day-old male Nicholas turkey poults were purchased from a commercial hatchery in California, Missouri. Poults were weighed, wing-banded, and sorted to a randomized block design in stainless steel battery pens. Housing was in an environmentally controlled room with a 24-hour constant light schedule. Feed and water were supplied *ad libitum*. Day-old poults were divided among seven dietary treatments, seven birds per pen, and seven replicate pens per treatment, and fed dietary treatments for 21 days. The animal care and use protocol was reviewed and approved by the University of Missouri Animal Care and Use Committee (ACUC).

On day 21, poults were euthanized using carbon dioxide, and weighed. Residual feed was measured and recorded. At termination, kidneys, livers, and serum were collected from three birds per pen. Response variables included feed intake at 21 days, body weight gain, feed conversion (F:G), mortality, relative kidney and liver weights, gross histopathology, and serum uric acid values.

Statistical Analyses

Data for all response variables were subjected to regression analysis using the general linear models procedure of SAS software (SAS Institute, 2006). Regression analysis best fit the means relative to a linear ($y_i = a + bx_i + E_i$), quadratic ($y_i = a + bx_i + cx^2_i + E_i$) or cubic ($y_i = a + bx_i + cx^2_i + dx^3_i E_i$) effect. Dunnett's test was run to calculate means among treatments and determine at which level of inclusion the specific parameter was significant from the control. Statistical significance was determined at a p-value less than or equal to 0.05.

Results

Mortality and Growth Performance

The effects of dietary treatments on mortality and growth performance are summarized in Table 7.2. No mortality was observed among any treatments. Although feed intake was different among treatments (P = 0.0468), there was no significant difference when treatments were compared to control using the Dunnett's test. Bird gain did not show a difference among treatments (P > 0.05). The ability of birds to convert feed to body weight gain (F:G) with increasing dietary concentrations of cyanuric acid also did not differ among treatments (P > 0.05). Additionally, no linear or quadratic regression was observed for these variables.

Organ Weights

Effects of dietary treatments on relative kidney and liver weights are summarized in Table 7.2. Relative weights of kidney and liver were not affected (P =

0.4908 and P = 0.1779, respectively) by increasing dietary concentrations of cyanuric acid.

Serum Chemistry

Serum uric acid values are presented in Table 7.2. Uric acid values were within normal range and no significance was found among treatments (P = 0.6807).

Pathology

Gross Pathology

Gross pathology of broilers fed 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0% cyanuric acid at termination of the experiment were found to be unremarkable.

Histopathology

Five kidney sections for each treatment were fixed and analyzed. All kidney sections were found to be unremarkable.

Discussion

In this study, mortality, body weight gain, and feed conversion were not different among treatments (P > 0.05). These results suggest that cyanuric acid included in the diet at up to 3% is not toxic. These data support the study by Dobson et al. (2008) in which individual triazines fed to rats resulted in no observable toxicity, and also confirm a previous study (Chapter 4) in which growth performance was not depressed in broilers fed cyanuric acid alone. In the present study, no mortality was observed, which corresponds to findings by Reimschuessel et al. (2008) where significant mortality was not reported in animals fed cyanuric acid alone. Only when cyanuric acid is combined

with melamine, has significant mortality occurred, due to renal failure in cats and dogs (Brown et al., 2007; Cianciolo et al., 2008). Due to the lack of difference among the control diet and the treatment diets, differences among treatments for feed intake may be best represented as a hormesis effect (Mattson, 2008); inclusion of cyanuric acid in low levels, such as 0.5%, caused an increase in feed intake, but inclusion of higher doses of cyanuric acid decreased feed intake comparable to normal control values.

In the present study, relative kidney weights were similar among all treatments and were unaffected by addition of cyanuric acid, whereas animals fed a combination of melamine and cyanuric acid were found to have an increased relative kidney weight (Cianciolo et al., 2008; Dobson et al., 2008). In the study by Cianciolo et al. (2008), it was found that addition of cyanuric acid to a melamine contaminated diet altered the elimination kinetics of melamine, causing crystals to precipitate in the renal tubules, but otherwise individually fed triazines, such as cyanuric acid, did not precipitate in the kidney (Dobson et al., 2008). Similar to the broiler research presented in Chapter 4, cyanuric acid alone does not cause toxic effects on liver or kidneys of turkey poults. This was found to be in contrast to our results with turkeys and broilers fed melamine alone, in which relative liver weights were increased with increasing levels of melamine. These results suggest that unlike melamine, the cyanuric acid compound is able to be degraded and/or excreted without harm to the poult's kidney or liver.

Gross examination of birds after three weeks of treatment revealed no toxic effect of cyanuric acid, and likewise, all kidney sections were unremarkable suggesting cyanuric acid alone does not cause renal pathology in turkeys fed levels ≤ 3%.

Furthermore, serum uric acid values were not affected by inclusion of cyanuric acid in levels up to 3% of the diet. These data are supported by Reimschuessel et al. (2008) in which no significant serum or pathological effects are observed in pigs fed up to 0.4% cyanuric acid.

Results of this study correspond to that which was presented earlier for broilers, indicating that cyanuric acid alone in concentrations up to 3.0% is not toxic to poults fed dietary treatments from hatch to day 21.

Table 7.1. Ingredient and nutrient composition of basal ration

Item	Composition (%)
Ingredient	
Soybean Meal	50.60
Corn	39.23
Corn Oil	2.77
Dicalcium	2.42
Limestone	1.14
Salt	0.39
Methionine	0.21
Trace Mineral[1]	0.10
Selenium Mix[2]	0.08
Vitamin Mix[3]	0.06
Copper Sulfate	0.004
Sand	3.00
Total	100
Nutrient composition (calculated)	
Crude Protein (%)	28.00
Metabolizable Energy (Kcal/kg)	2800
Lysine (%)	1.60
Methionine (%)	0.62
Methionine + Cysteine (%)	1.05
Threonine (%)	1.06
Calcium (%)	1.20
Phosphorus (% Av.)	0.60

[1]Trace mineral mix provided (mg/kg of diet): manganese, 110 mg from $MnSO_4$; iron, 60 mg from $FeSO_4 \bullet 7H_2O$; zinc, 110 mg from $ZnSO_4$; iodine, 2 mg from ethylenediamine dihydroidodide.
[2]Selenium premix provided 0.2 mg of Se/kg of diet from $NaSeO_3$.
[3]Vitamin mix supplied (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 7.2. Effects of cyanuric acid on performance of turkey poults[1]

	Treatments[2] – Cyanuric acid %							ANOVA[6]		Regression[7]	
Item	0	0.5	1.0	1.5	2.0	2.5	3.0	Pooled SEM	P-value	L/Q[5]	P-value
FI, g	747	826	811	756	803	745	745	23	0.0468	-	0.1534
BWG, g	434	466	448	421	434	424	427	15	0.3889	-	0.1482
F:G	1.751	1.781	1.814	1.800	1.862	1.771	1.750	0.07	0.9026	-	1.0000
Organ Weight[3]											
Kidney	0.97	1.00	0.99	0.99	1.04	1.02	0.98	0.04	0.4908	-	0.4908
Liver	2.91	2.73	2.91	2.78	2.89	3.11	2.83	0.14	0.1779	-	0.3507
Mort[4] %	0	0	0	0	0	0	0	-	-	-	-
Serum UA[5] (mg/dL)	7.34	6.23	6.24	6.54	5.62	6.10	7.07	1.09	0.7423	-	0.6807

[1]Data are means of 25 birds per treatment for growth performance parameters and 15 birds per treatment for relative organ weight and serum uric acid levels.
[2]0 = Basal diet (BD); 0.5 = BD + 0.5% CA; 1.0 = BD + 1.0% CA; 1.5 = BD + 1.5% CA; 2.0 = BD + 2.0% CA; 2.5 = BD + 2.5% CA; 3.0 = BD + 3.0% CA.
[3]Relative organ weights expressed as a percentage of body weight.
[4]Mort = Mortality %
[5]UA = serum uric acid
[6]One way analysis of variance values
[7]Regression- L = linear regression and Q = quadratic regression

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CHAPTER 8

Individual and Combined Effects of Melamine and Cyanuric Acid in Turkey Poults

Introduction

Concerns regarding adulteration of protein ingredients used in pet foods and human foods with melamine (M) have resulted in the recall of food and feed products. Upon further investigation, it was determined that a combination of melamine and cyanuric acid (CA) was responsible for toxic effects in animals and humans (Brown et al., 2007). Melamine and cyanuric acid are triazine compounds that are normally used as chemicals in production of fire retardant materials and pool chlorine stabilizers, respectively (Lowy and Pritchard, 2008; Cantu et al., 2001). Melamine (1,3,5-triazine-2,4,6-triamine) can be degraded by removing three NH_2 groups and replacing them with OH groups to form cyanuric acid (1,3,5-triazine-2,4,6-trihydroxy; Jutzi et al., 1982).

On March 16th 2007, Menu Foods placed a recall on wheat gluten and rice protein products imported from the Chinese food company, ChemNutra (Burns, 2007a; FDA 2007b). The contaminated products were recalled and instructed to be destroyed (FDA, 2007b). From April 5 to June 6, 2007, 235 cats and 112 dogs (mortality was 61% and 74%, respectively) were diagnosed with "pet food-induced nephrotoxicity" based on high concentrations of blood urea nitrogen and creatinine (Burns, 2007b). Studies,

such as those by Brown et al. (2007), found that melamine and cyanuric acid were present in renal tissues of affected animals. "Spoke-like" crystals, formed from the consumption of melamine and cyanuric acid, affected animal kidneys and were suspected to compromise kidney function, leading to acute renal failure and death in cats and dogs. Affected animals also developed lesions such as oral ulcers along the tongue and mineralization in the gastric mucosa, pulmonary smooth muscle and alveolar walls (Brown et al., 2007; Cianciolo et al., 2008).

Furthermore, pigs and chickens consuming melamine and cyanuric acid contaminated pet food byproducts were traced to various states across the nation (Burns, 2007a; FDA, 2007a). No mortality was reported in the pigs and chickens fed these products, and research by Filigenzi et al. (2007) determined that residues in the tissue of the affected livestock animals were not high enough to pose a threat to human health. Upon further investigation, it was determined that cyanuric acid alone can not be metabolized in mammals, and therefore is generally excreted via urine (Dobson et al., 2008; Filigenzi et al., 2007), unlike melamine, which has been found to accumulate in the renal tubules as crystals (Reimschuessel et al., 2008).

Combinations of melamine and its triazine derivatives such as cyanuric acid were studied in various animals. Puschner et al. (2007) found that cats fed combinations of high concentrations of melamine and cyanuric acid had severe kidney lesions, and microscopic, green-brown, circular crystals were found in the kidneys of cats fed a combination of 1% melamine and 1% cyanuric acid. Combinations of melamine and cyanuric acid in a 400/400 mg/kg/day mixture were considered toxic to rats (Jingbin et

al., 2010). Likewise, toxicity was reported in rats fed melamine, ammeline, ammelide and cyanuric acid in a 400/40/40/40 mg/kg/day mixture (Dobson et al., 2008). Research with fish fed melamine and cyanuric acid in combination resulted in white feces and excretion of nitrogenous waste via the gills of trout, salmon and catfish, and death of two fish in the study (Reimschuessel et al., 2008). Reimschuessel continued research in pigs and reported edema of the fascia around the kidneys of pigs administered dietary melamine and cyanuric acid in a 1:1 combination. Creatinine and blood urea nitrogen levels were elevated in affected swine, although not significantly different from control pigs.

The objective of this study was to determine the individual and combined effects of melamine and cyanuric acid in turkey poults fed dietary treatments from hatch to 21 days.

Materials and Methods

Diet Preparation

Ten dietary treatments were prepared using graded levels of melamine and cyanuric acid purchased from Sigma-Aldrich Chemical Company (St. Louis, Mo.). A control diet (basal diet) was formulated to meet or exceed requirements of turkey poults as recommended by the National Research Council (NRC, 1994; Table 8.1). Three of the diets contained melamine alone at inclusion levels of 0.5, 1.0, and 1.5%. Another three diets contained cyanuric acid alone at inclusion levels of 0.5, 1.0, and 1.5%. The remaining three diets consisted of combinations of melamine and cyanuric acid in 1:1

ratio totaling 1.0, 2.0, and 3.0% of the diet. Melamine and cyanuric acid were substituted for sand (up to 3.0%) in the basal diet.

Birds, Management and Response Variables

Two hundred and fifty day-old male Nicholas turkeys were purchased from a commercial hatchery. Poults were weighed, wing-banded, and sorted to a randomized block design in stainless steel battery pens. Housing was in an environmentally controlled room with a 24-hour constant light schedule. Feed and water were supplied *ad libitum*. Day-old poults were divided among 10 dietary treatments, five birds per pen, and five replicate pens per treatment, and fed dietary treatments for 21 days. The animal care and use protocol was reviewed and approved by the University of Missouri Animal Care and Use Committee (ACUC).

On day 21, poults were euthanized using carbon dioxide, and weighed. Poults that died before termination were weighed and sent to a pathology lab for necropsy. Blood samples were collected via cardiac puncture from 15 anesthetized birds per treatment and serum was separated for analysis of serum chemistries. Residual feed was measured and recorded. At termination, kidneys and livers were collected from three birds per pen to determine weights relative to final body weight. In addition, samples of kidney were collected from five birds per treatment for histopathologic examination. Response variables included growth performance at 21 days (feed intake, body weight gain, and feed conversion), mortality, serum chemistry, relative kidney and liver weights, gross pathology, and histopathology of fixed kidney sections.

Statistical Analyses

Data for all response variables were analyzed by the general linear model procedure of SAS software (SAS Institute, 2006) as a 3 x 3 factorial plus an additional treatment. Individual means and interaction means were calculated for melamine and cyanuric acid treatments. Means for treatment showing significant differences in the main effect means were compared using Duncan's Multiple Range Test procedure. Statistical significance was determined at a p-value less than or equal to 0.05.

Results

Mortality and Growth Performance

The individual and combined effects of melamine and cyanuric acid on mortality and growth performance are summarized in Table 8.2. There was an effect ($P < 0.05$) of dietary treatments on mortality. Compared to controls, 56% of birds fed 1.5% melamine died before 21 days of age, followed by 16% of birds fed 1.0% cyanuric acid, and 8% of birds fed the 3.0% combination (1.5% melamine and 1.5% cyanuric acid) diet. An interactive effect between melamine and cyanuric acid was observed due to changes in mortality differing in poults fed melamine alone, cyanuric acid alone, and the combination of melamine and cyanuric acid. Higher mortality ($P < 0.05$) was observed in birds fed 1.5% melamine compared to birds fed 0.5 or 1.0% (32 vs 4 and 0%, respectively) melamine. There was no mortality among birds fed 0.5 or 1.5% cyanuric acid.

Compared to controls, feed intake (FI) and body weight gain (BWG) were decreased ($P < 0.0001$) in birds fed diets containing melamine alone (FI, 772 vs 874 g;

BWG, 522 vs 647 g). In contrast, compared to controls, FI and BWG were only marginally decreased in birds fed cyanuric acid alone (FI, 819 vs 874 g; BWG, 631 vs 647 g). The largest decrease in feed intake and body weight gain was observed in birds fed 1.5% melamine (FI was 307 g less than controls, and BWG was 207 g less than controls). No interactive effect was found between melamine and cyanuric acid ($P > 0.05$) for body weight gain, although an interactive effect between melamine and cyanuric acid was observed for FI ($P = 0.0374$). This interaction occurred because changes in FI differed in birds fed melamine alone, cyanuric acid alone, and the combination of melamine and cyanuric acid. There were main effects of melamine alone ($P < 0.0001$), cyanuric acid alone ($P = 0.0014$), and an interactive effect between melamine and cyanuric acid ($P = 0.0150$) in the ability of birds to convert feed to body weight gain (F:G). Birds fed 1.5% melamine alone had poorer F:G ($P < 0.05$) than birds fed 0.5 or 1.0% melamine, whereas there was no difference in F:G among birds fed 0.5, 1.0, or 1.5% cyanuric acid.

Organ Weights

Effects of dietary treatments on relative kidney and relative liver weights are summarized in Table 8.2. Compared to controls, poults fed melamine alone had heavier relative kidney weights (1.25 vs 0.94%) with the heaviest kidney weights as a percentage of body weight observed in birds fed 1.5% melamine (1.422%). Compared to controls, poults fed cyanuric acid alone had similar relative kidney weights (0.96 vs 0.94%). Main effect differences in relative kidney weights were observed for both melamine and cyanuric acid ($P < 0.0001$). Birds fed 1.5% melamine had heavier relative kidney weights (1.20%) compared with birds fed 0.5 and 1.0% melamine. By contrast, there was no

difference in relative kidney weights among birds fed 0.5, 1.0, or 1.5% cyanuric acid. This difference in response between melamine and cyanuric acid for relative kidney weights resulted in a melamine by cyanuric acid interaction (P = 0.0011).

A main effect difference in relative liver weights was observed in birds fed cyanuric acid with birds fed no cyanuric acid having heavier liver weights relative to body weight than birds fed cyanuric acid (P = 0.0002). However, this difference appeared to occur because of the numerical increase in relative liver weights in birds fed diets containing melamine alone instead of an increase due to cyanuric acid. There was no effect of melamine (P > 0.05) on relative liver weights nor was there an interaction between melamine and cyanuric acid for relative liver weight (P > 0.05).

Serum Chemistry

Effects of treatments on various serum values are presented in Table 8.3. A main effect of melamine was observed for serum albumin (ALB) levels (P = 0.0417), and a main effect of cyanuric acid was observed for serum calcium (CA) levels (P = 0.0125). A main effect mean of melamine alone and cyanuric acid alone was observed for serum aspartate transaminase (AST) values (P = 0.0225 and P < 0.0001, respectively). Increasing levels of dietary melamine caused an increase in levels of AST, whereas increasing levels of dietary cyanuric acid caused a decrease in serum AST levels. The combination of melamine and cyanuric acid resulted in an interactive effect for serum uric acid values in turkey poults (P = 0.0080).

Pathology

Gross Pathology - Early Mortality

Fourteen of fifteen poults fed 1.5% melamine alone died with gross lesions compatible with melamine toxicity (accumulation of spherical, basophilic crystals in the collecting ducts and collecting tubules). One poult from the 3.0% melamine and cyanuric acid combination treatment died with gross lesions compatible with melamine toxicity. One of four poults fed 1.0% cyanuric acid had visibly pale kidneys. The remaining six poults (one fed 0.5% melamine, three fed 1.0% cyanuric acid, one fed the 1.0% melamine and cyanuric acid combination diet, and one fed the 3.0% melamine and cyanuric acid combination diet) that died before termination died of miscellaneous causes: no gross lesions, cannibalism, tibial rotation, and right sided heart failure.

Histopathology-– Early Mortality

Fifteen of fifteen kidney sections from poults fed 1.5% melamine had moderate to severe autolysis with basophilic mineralized casts in the renal tubules, collecting tubules, and collecting ducts. These lesions were characteristic for melamine toxicity. Four of four kidney sections from poults fed 1.0% cyanuric acid had moderate autolysis with no other lesions. One kidney from a poult fed the 3.0% melamine and cyanuric acid combination had moderate autolysis but was otherwise unremarkable. One kidney from a poult fed the 3.0% melamine and cyanuric acid combination had moderate autolysis with basophilic mineralized casts in the renal tubules, collecting tubules, and collecting ducts. These lesions were characteristic for melamine toxicity. Neither kidney section from poults fed the 3.0% combination of melamine and cyanuric acid contained polarizable crystals, as seen with melamine and cyanuric acid crystal complexes.

Histopathology - Termination of Experiment

One of six kidney sections from poults fed 1.0% melamine, and five of six kidney sections from poults fed 1.5% melamine revealed mild focal interstitial heterophil infiltration in the medullary cone region. All other fixed kidney sections, including those where poults were fed combinations of melamine and cyanuric acid, were deemed unremarkable. To better evaluate the kidney sections, the six sections of poults fed the combination of 1.5% melamine and 1.5% cyanuric acid were examined under polarized light to detect the characteristic polarizable melamine/cyanuric acid crystals but no such crystals were observed.

Discussion

The 56% mortality observed in poults fed 1.5% melamine alone is similar to a previous study (Chapter 6) where mortality was 32% in poults fed 1.5% melamine. In the previous study, 67% of treatment related deaths occurred within the first 10 days of treatment, and likewise 92% of the mortality observed in the current study occurred in the first 10 days of treatment in birds fed 1.5% melamine alone. Similarly, 75% of the mortality observed in broilers (Chapter 3) fed melamine alone was observed in the first 10 days of treatment.

Reduced FI and BWG were observed in broilers and turkeys fed melamine alone (Chapters 3 and 6), but were not evident in broilers or turkeys fed cyanuric acid alone (Chapters 4 and 7). Previous work (Chapter 3, 5 and 6) had suggested that melamine was not toxic until birds (turkeys and broilers) were fed at least 1.0% melamine, but this study found that inclusion of 0.5% melamine in the diet decreased FI and BWG. While

0.5% melamine alone caused a decrease, the largest depression in FI and BWG, compared to controls, were observed in poults fed 1.5% melamine (307 gram reduction in FI, and 207 gram reduction in BWG; ($P < 0.05$). These reductions correlate to the high mortality rate which occurred in poults fed 1.5% melamine. While the lowest level of melamine in this study (0.5%) reduced FI and BWG in poults, this level of melamine did not cause a depression in FI and BWG in broilers (Chapter 3) turkeys (Chapter 6) or ducks (Landers et al., 2010). In the only other study reporting the effects of graded doses of melamine in chicks, Lu et al. (2009) observed no negative effects on growth performance of broilers fed up to 0.1% melamine, a level five-fold lower than the lowest level fed in the current study.

The decreased FI and BWG observed in poults fed 0.5 and 1.0% cyanuric acid alone was surprising since depressions in FI and BWG were not observed in a previous study in which turkeys were fed ≥ 3% cyanuric acid (Chapter 7). Even more surprising was the fact that birds fed 1.5% cyanuric acid performed as well as birds fed the control diet. Cyanuric acid at concentrations up to 3.0% also did not affect FI or BWG in broilers fed dietary treatments for three weeks (Chapter 4).

Interestingly, the addition of cyanuric acid to the melamine diets appeared to reduce the negative effects of melamine on FI and BWG. Birds fed the combination diets consumed considerably more feed (on average 845 vs 698 grams) and gained considerably more weight (on average 636 vs 522 grams) than birds fed melamine alone. A similar response was not observed in a previous study (Chapter 5) in which broilers were fed identical dietary treatments.

The main effect differences and the interaction between melamine and cyanuric acid for F:G was not observed in a previous study in which chicks were fed identical dietary treatments (Chapter 5). Similarly, changes in F:G were not observed in turkeys fed up to 3.0% cyanuric acid (Chapter 7) or up to 3.0% melamine (Chapter 6). Similar to the response observed for FI and BWG, cyanuric acid appeared to ameliorate the effects of melamine on F:G with birds fed the combination diets being more efficient in converting feed to gain compared to those fed melamine alone (on average 1.38 vs 1.63 g:g).

Increased relative kidney weights found in birds fed melamine alone (on average 1.25 vs 0.94 g:g for controls) has been observed previously in broilers (Chapters 3 and 5), turkeys (Chapter 6), and ducks (Landers et al., 2010) fed melamine alone and is consistent with previous reports indicating that the kidney is the target organ for melamine. In comparison to melamine, cyanuric acid alone caused a relatively small increase in relative kidney weights (on average 0.96 vs 0.94 g:g for controls) compared to controls. However, similar to growth performance, the addition of cyanuric acid to the melamine diets appeared to ameliorate the effects of melamine on relative kidney weights with relative kidney weights of birds fed the combination diets averaging 0.99 g:g compared to 1.25 g:g for birds fed melamine alone.

Increasing serum albumin levels with increasing dietary melamine concentrations was not observed in a previous study in which turkeys were fed up to 3.0% melamine (Chapter 6). A decrease in serum calcium was observed in poults fed levels up to or equal to 1.0% cyanuric acid, but then were returned to normal control

values when fed 1.5% cyanuric acid. Changes in serum calcium were also noted in broiler chicks fed 3.0% melamine (Chapter 3), in which calcium levels were higher than controls. Similar to previous studies with chicks (Chapter 3 and 5), increasing levels of melamine alone caused increases in serum aspartate transaminase values, whereas increasing levels of cyanuric acid alone caused a decrease in serum AST levels. Unlike the previous chapter with broilers (Chapter 6), an interactive effect between melamine and cyanuric acid was observed in serum uric acid values. A change in serum uric acid levels correlates to what was found in other studies: blood urea nitrogen levels were elevated in animals fed melamine and cyanuric acid combinations (Brown et al., 2007; Burns, 2007b; Cianciolo et al., 2008). Increasing levels of nitrogen circulating in the blood signify that the body is having difficulty ridding metabolic wastes and the increase in blood urea nitrogen was used as an indicator of renal failure in affected animals. However, the present study suggests that addition of cyanuric acid to melamine contaminated diets actually alleviates the toxic effect of melamine. While changes in serum chemistry values were observed, all serum values fell within or near normal expected values for poultry species (Braun et al., 1982; Coles, 1985; Coles, 1997; and Puls, 1994).

Lesions observed in kidneys of poults fed 1.5% melamine alone exhibited similar pathology to that observed in broilers (Chapters 3 and 5), turkeys (Chapter 6), and ducks (Landers et al., 2010) fed melamine alone. The addition of 1.5% cyanuric acid to the 1.5% melamine diet (1.5% combination) seemed to protect the kidney from the effects of melamine when fed alone since only one poult in this treatment exhibited lesions

characteristic of melamine toxicity. The lesions caused by melamine and cyanuric acid crystalline complexes precipitating in the kidney that were observed in broilers (Chapter 5), cats (Brown et al., 2007), dogs (Puschner et al., 2007), rats (Cianciolo et al., 2008), and fish (Reimschuessel et al., 2008) were not observed in the current study with poults. The mild interstitial nephritis noted primarily in birds fed 1.5% melamine is probably a real treatment associated lesion, considering this lesion was not observed in the controls or cyanuric acid treatments. The severity of this lesion was mild and focal and would not compromise renal function. In this experiment, birds fed 1.5% melamine had the highest mortality with the characteristic gross and microscopic lesions of melamine toxicity. In the kidney sections collected from poults on this treatment that survived until the termination of the three week trial, it is likely that the mild nephritis is the recovery phase of mild to moderate melamine associated lesions that occurred at the beginning of the experiment.

One might have expected birds fed the 3.0% combination of melamine and cyanuric acid to have a similar mild nephritis but the addition of cyanuric acid appeared to have a protective effect against this lesion. This finding correlates with the high mortality observed in birds fed 1.5% melamine alone and the low mortality observed in birds fed a combination of 1.5% melamine and 1.5% cyanuric acid. Poults seem to be more efficient at excreting the melamine-cyanuric acid complexes than broilers when comparing histopathology of those fed combinations up to 3.0%. Moreover, mammals appear to be less efficient at clearing melamine and cyanuric acid complexes than poultry, considering mammals were fed much lower levels of melamine and cyanuric

acid than poults in the current study or broilers in a previous study (Chapter 5). One explanation for this increased excretory efficiency may be the finding that birds appear to be capable of excreting melamine via the bile; a finding not reported in mammals.

Results of the current study indicate that of the two triazines, melamine is more toxic than cyanuric acid. Cyanuric acid appears to ameliorate the effects of melamine reducing not only its negative effects on growth performance and relative kidney weights but also preventing the formation of kidney lesions caused by melamine crystals. In addition, the combinations of melamine and cyanuric acid did not result in melamine-cyanuric acid crystal formation in the kidneys of turkeys as was observed previously in broilers (Chapter 5), cats (Puschner et al., 2007), dogs (Brown et al., 2007), and fish (Reimschuessel et al., 2008). In conclusion, the combination of melamine and cyanuric acid was less toxic to poults than melamine alone, and poults were able to survive to 21 days of age without significant toxic effects.

Table 8.1. Ingredient and nutrient composition of basal ration

Item	Composition (%)
Ingredient	
Soybean Meal	50.60
Corn	39.23
Corn Oil	2.77
Dicalcium	2.42
Limestone	1.14
Salt	0.39
Methionine	0.21
Trace Mineral[1]	0.10
Selenium Mix[2]	0.08
Vitamin Mix[3]	0.06
Copper Sulfate	0.004
Sand	3.00
Total	100
Nutrient composition (calculated)	
Crude Protein (%)	28.00
Metabolizable Energy (Kcal/kg)	2800
Lysine (%)	1.60
Methionine (%)	0.62
Methionine + Cysteine (%)	1.05
Threonine (%)	1.06
Calcium (%)	1.20
Phosphorus (% Av.)	0.60

[1]Trace mineral mix provided (mg/kg of diet): manganese, 110 mg from $MnSO_4$; iron, 60 mg from $FeSO_4 \bullet 7H_2O$; zinc, 110 mg from $ZnSO_4$; iodine, 2 mg from ethylenediamine dihydroidodide.
[2]Selenium premix provided 0.2 mg of Se/kg of diet from $NaSeO_3$.
[3]Vitamin mix supplied (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 8.2. Effects of melamine and cyanuric acid on performance of turkey poults[1]

Melamine (%)	Cyanuric Acid (%)	FI (g)	BWG (g)	F:G	Relative Kidney Weight[2]	Relative Liver Weight[2]	Mortality (%)
0	0	874	647	1.350	0.940	2.57	0
0.5	0	767	572	1.390	1.081	2.64	4
1.0	0	760	553	1.382	1.239	2.88	0
1.5	0	567	440	2.129	1.422	2.81	56
0	0.5	826	615	1.337	0.966	2.63	0
0	1.0	772	604	1.425	0.946	2.56	16
0	1.5	860	674	1.271	0.963	2.66	0
0.5	0.5	823	635	1.375	1.005	2.64	4
1.0	1.0	846	633	1.337	0.929	2.54	0
1.5	1.5	867	642	1.422	1.028	2.64	8
Pooled SEM[3]		31	19	0.094	0.06	0.09	4
Source of Variation		----------P----------					
Melamine (M)		0.0009	0.0001	<.0001	<.0001	0.1224	<.0001
Cyanuric Acid (CA)		<.0001	<.0001	0.0014	<.0001	0.0002	<.0001
M x CA		0.0374	0.2367	0.0150	0.0011	0.0891	0.0002
Main Effect Means							
Melamine	0	819[a]	631[a]	1.344[b]	0.96[c]	2.62	5[b]
	0.5	795[a]	604[a]	1.383[b]	1.04[b]	2.64	4[b]
	1.0	803[a]	593[a]	1.360[b]	1.08[b]	2.71	0[b]
	1.5	717[b]	541[b]	1.776[a]	1.20[a]	2.71	32[a]
Cyanuric Acid	0	698[b]	522[c]	1.634[a]	1.23[a]	2.77[a]	20[a]
	0.5	825[a]	625[ab]	1.356[b]	0.99[b]	2.64[b]	2[b]
	1.0	809[a]	619[b]	1.381[b]	0.94[b]	2.55[b]	8[b]
	1.5	863[a]	658[a]	1.347[b]	1.00[b]	2.65[b]	4[b]

[1]Data are means of 25 birds per treatment for growth performance parameters and 15 birds per treatment for organ weights.
[2]Relative organ weights expressed as a percentage of body weight.
[3]One way analysis of variance values
[a-c]Means with different superscripts in a column differ significantly ($P < 0.05$)

Table 8.3. Serum chemistries of turkey poults fed graded levels of melamine and cyanuric acid[1]

Melamine (%)	Cyanuric Acid (%)	GLU (mg/dL)	ALB (g/dL)	TP (g/dL)	GLOB (g/dL)	CA (mg/dL)	AST (IU/L)	UA (mg/dL)
0	0	306	1.27	2.91	1.64	8.63	244	5.12
0.5	0	292	1.19	2.78	1.59	7.81	250	4.43
1.0	0	312	1.25	2.87	1.62	8.00	287	5.74
1.5	0	330	1.31	2.95	1.64	8.27	313	6.40
0	0.5	300	1.29	2.99	1.70	7.95	257	4.12
0	1.0	300	1.21	2.83	1.62	7.09	254	4.88
0	1.5	299	1.31	3.01	1.70	8.10	235	4.54
0.5	0.5	305	1.23	2.86	1.63	6.18	252	5.49
1.0	1.0	292	1.20	2.83	1.63	6.91	232	4.07
1.5	1.5	302	1.24	2.85	1.61	7.65	228	4.26
Pooled SEM[2]		14	0.04	0.10	0.60	0.62	17	0.73
Source of Variation								
Melamine (M)		0.1878	0.0417	0.1816	0.2581	0.0772	0.0225	0.6075
Cyanuric Acid (CA)		0.2967	0.0772	0.3247	0.4857	0.0125	<.0001	0.1070
M x CA		0.2098	0.1605	0.3336	0.3183	0.4492	0.0643	0.0080
Main Effect Means								
Melamine	0	300	1.27[ab]	2.94	1.67	7.71	249	4.51
	0.5	298	1.21[b]	2.82	1.61	7.00	251	4.96
	1.0	302	1.23[ab]	2.85	1.63	7.46	259	4.91
	1.5	316	1.28[a]	2.90	2.30	7.96	271	5.33
Cyanuric Acid	0	311	1.25	2.87	2.07	8.03[a]	283[a]	5.52
	0.5	303	1.26	2.93	1.67	7.07[b]	254[b]	4.81
	1.0	296	1.21	2.83	1.63	7.00[b]	243[b]	4.48
	1.5	301	1.28	2.93	1.66	7.88[ab]	232[b]	4.40

[1]Data are means of 15 birds per treatment: GLU = glucose, ALB = albumin, TP = total protein, GLOB = globulin, CA = calcium, AST = aspartate transaminase, UA = uric acid.
[2]One way analysis of variance values
[a-b]Means with different superscripts in a row differ significantly ($P < 0.05$).

CHAPTER 9

CONCLUSION

Six experiments were conducted to investigate the effects of melamine and cyanuric acid when fed alone or in combinations less than or equal to 3% to young broilers and turkey poults. In experiment 1 (Chapter 3), day old broilers were fed diets supplemented with 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0% melamine for 21 days. Mortality was significant for birds fed greater than or equal to 2.5% melamine. Compared to controls, feed intake and body weight gain were significantly depressed in birds fed greater than or equal to 1.0% melamine. Compared to controls, feed to gain conversion was worsened in birds fed greater than or equal to 2.5% melamine. Relative kidney weights increased linearly, and were higher in birds fed greater than or equal to 1.5% melamine. Relative liver weights increased quadratically, and were higher than controls when birds were fed greater than or equal to 2.5% melamine. Melamine concentrations were greatest in the kidney of birds (quadratic effect), followed by liver (linear effect), bile (quadratic effect), and breast muscle (linear effect). The significant levels of melamine quantified in the bile suggest that biliary excretion may be beneficial to broilers in preventing toxic effects of melamine at these high dietary concentrations. Non-polarizable melamine crystals, similar to those observed in other studies, were found to cause renal pathology in collecting tubules and ducts. This finding is further

supported by the visual observance of pale, enlarged kidneys in affected birds, especially those that died due to renal failure. Additionally, birds fed greater than or equal to 1.0% melamine may contain enough melamine residue to exceed the tolerable daily intake (TDI) of 0.63 mg/kg BW/day, assuming a 63 kg person consumes 454 grams of melamine contaminated breast muscle in one day. However, it is not practical to suggest that melamine would be included at such high levels, considering the initial contaminations in 2007 were exceedingly lower than what was observed in this experiment.

Experiment 2 (Chapter 4) tested the effects of 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0% cyanuric acid fed to broiler chicks for 21 days. Morality, feed intake, body weight gain, and feed to gain conversion were not significant among treatments for birds fed up to 3.0% cyanuric acid. Likewise, relative liver weight and relative kidney weight were not affected by increasing dietary concentrations of cyanuric acid. Upon necropsy and analysis of bile, birds and samples were found to be unremarkable, including no observation of crystals in the bile. These data suggest that cyanuric acid in levels less than or equal to 3.0% is not toxic to broiler chicks. Lack of toxicity, in comparison to broilers fed melamine, may be attributed to the properties of the hydroxyl groups of the cyanuric acid compound, which replace the amine groups of melamine. Weak hydroxyl groups may prevent cyanuric acid compounds from bonding, reducing crystal formation and blockage in the kidneys.

Experiment 3 (Chapter 5) evaluated the individual and combined effects of melamine and cyanuric acid fed to young broiler chicks. Ten dietary treatments were

evaluated: control, melamine alone at 0.5, 1.0, and 1.5% inclusion, cyanuric acid alone at 0.5, 1.0, and 1.5% inclusion, and combinations of melamine and cyanuric acid in a one to one ratio totaling 1.0, 2.0, and 3.0% inclusion. Mortality was not significant among dietary treatments. Feed intake and body weight gain significantly decreased as levels of melamine alone increased, but were not affected by increasing levels of cyanuric acid alone. An interactive effect between melamine and cyanuric acid was observed for feed intake and body weight gain: birds, on average, consumed 104 grams less feed than controls and gained 85 grams less than control birds. Feed conversion was not significant among treatments. Chicks fed melamine alone or combinations of melamine and cyanuric acid had heavier kidney weights as a percentage of body weight (especially birds fed 1.5% melamine alone or in combination) than birds fed the control diet. Birds fed cyanuric acid alone had heavier relative liver weights than controls, and an interactive effect between melamine and cyanuric acid was observed for relative liver weights. The only observed significant difference in serum values was for serum aspartate transaminase levels, with serum aspartate transaminase levels increasing with increasing concentrations of melamine, and decreasing with increasing cyanuric acid levels. All serum values, including that of serum aspartate transaminase, fell within or near normal expected values for poultry. A total of seven out of 15 observed kidney sections from the combinations diets (1.0, 2.0, and 3.0%) were found to have green, spherical, spirally radiating crystals in the collecting tubules and ducts. Kidney sections of birds fed individual triazines remained unremarkable. This suggests that melamine

and cyanuric acid complex into a polarizable crystal that potentially accumulates in the kidney of affected animals.

In experiment 4 (Chapter 6), day old turkeys were fed diets supplemented with 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0% melamine for 21 days. Significant mortality within the first 10 days resulted in early termination of poults fed greater than or equal to 2.0% melamine, and growth and performance data, and serum chemistries are not available for these treatments. Mortality was linearly related to increasing levels of dietary melamine up to 1.5%. Feed intake and body weight gain decreased linearly, and feed efficiency was notably worsened with increasing concentrations of melamine. Relative kidney weights and relative liver weights increased linearly with increasing dietary levels of melamine. No significant effect was observed among treatments for serum values, and values fell within or near normal expected values for poultry. Kidney melamine residue concentrations increased linearly, whereas breast muscle melamine residue concentrations increased quadratically with increasing dietary levels of melamine. Melamine residue levels in breast muscle were significantly higher than controls in birds fed greater than or equal to 1.0% melamine, whereas kidney melamine residue levels were significant in all birds fed melamine (0.5 to 3.0% melamine). Of those that died early in the study, gross pathology characteristic of melamine toxicity was observed in poults fed levels as low as 1.0% melamine. The pathology observed included pale, enlarge kidneys, crystals in the bile and on the liver/pericardium, and renal hemorrhaging. Eosinophilic to basophilic mineralized casts were observed in renal collecting tubules and ducts of kidneys of birds that died early in the study. Of the

poults that survived to 21 days of age, body size was the most notable difference among treatments, but gross examinations were otherwise unremarkable. When kidney sections were further evaluated post termination (21 days for birds fed less than or equal to 1.5% melamine, and 14 days of age for birds fed 2.0 to 3.0% melamine), mild interstitial heterophil infiltration was noted in birds fed greater than or equal to 1.0% melamine. While lesions were noted in those that survived to termination, they were considered mild and would not inhibit normal renal function.

Experiment 5 (Chapter 7) tested the effects of 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0% cyanuric acid fed to turkey poults for 21 days. No mortality was observed among treatments. Feed intake was significant among treatments but the Dunnett's test revealed no differences. Body weight gain and feed conversion were not significantly different among treatments. Likewise, relative liver weights and relative kidney weights were not affected by increasing dietary concentrations of cyanuric acid. Uric acid was the only serum value evaluated and no significant changes were found among treatments and values were within normal range for poultry. Gross pathology and histopathology were found to be unremarkable among all treatments. These data suggest that cyanuric acid in levels less than or equal to 3.0% is not toxic to turkey poults.

Experiment 6 (Chapter 8) explains the individual and combined effects of melamine and cyanuric acid fed to turkey poults. Ten dietary treatments were evaluated: control, melamine alone at 0.5, 1.0, and 1.5% inclusion, cyanuric acid alone at 0.5, 1.0, and 1.5% inclusion, and combinations of melamine and cyanuric acid in a one

to one ratio totaling 1.0, 2.0, and 3.0% inclusion. Birds fed 1.5% melamine alone had significant mortality (56%) before reaching 21 days of age, followed by 16% of birds fed 1.0% cyanuric acid alone and then 8% birds fed the 3.0% combination diet. An interactive effect between melamine and cyanuric acid was observed. Feed intake and body weight gain were depressed in birds fed melamine alone and an interactive effect was observed between melamine and cyanuric acid for feed intake. Main effects of melamine and cyanuric acid and interaction effects were noted in feed conversion. Poults fed melamine alone had significantly heavier kidney weights relative to body weight and main effects were observed for both melamine and cyanuric acid alone, whereas relative kidney weights of birds fed cyanuric acid alone were similar to control values, resulting in a melamine by cyanuric acid interaction. Significantly heavier liver weights as a percentage of body weight were observed in birds fed cyanuric acid compared to controls, but there was no effect of melamine alone, and no interaction effect for relative liver weights. Pale kidneys were observed in one poult fed 1.0% cyanuric acid that died before 21 days of age. Observations of the birds fed 1.5% melamine alone reveal accumulation of crystals in the collecting ducts and tubules of kidneys, characteristic of melamine toxicity. Early mortality birds fed the 3.0% combination diet revealed mineralized casts within the renal tubules, but crystals were not polarlizable. At termination, birds fed 1.0 and 1.5% melamine were found to have renal pathology associated with melamine toxicity, yet no kidney sections revealed polarized melamine-cyanurate crystals in birds fed combination diets.

Differences in poultry and companion animal response to melamine and cyanuric acid may be explained by the different feed processing techniques utilized for livestock animals compared to companion animals. In these six studies, the feed ingredients were mixed into a ration and were not subjected to heating or extruding. In contrast, companion animal diets generally are processed under extreme heat, which may affect the interaction of melamine and cyanuric acid in the feed. Changes in pH, especially in wet food products, may also initially alter the interaction and individual effects of melamine and cyanuric acid in companion animal feeds.

Physiological differences between poultry species and companion animals may also justify why dietary combinations of melamine and cyanuric acid were less toxic in birds than companion animals. Changes in the pH levels in birds, due to presence of a crop, proventriculus, and gizzard, may alter crystal formation of melamine and cyanuric acid in the gastrointestinal tract compared to what was observed in companion animals. It is hypothesized that the crystal complex forming in the gastrointestinal tract can not be absorbed into the blood stream and furthermore is not deposited in the kidneys. However, because melamine and cyanuric acid were added as individual powders in these six poultry diets, small concentrations of the individual triazines may be passed through the blood and into the kidneys, where they are concentrated and can further develop into melamine-cyanurate crystals. This suggests that most crystals forming in the gastrointestinal tract would be excreted, and therefore the toxic effects associated with melamine alone are less severe when cyanuric acid is included in the diet.

Results of these studies confirm previous reports that the kidney is the primary target organ affected by melamine and combinations of melamine and cyanuric acid. High residue levels of melamine in the kidney and detection of crystals in the collecting tubules and ducts support this observation. Furthermore, melamine crystals can be differentiated from melamine-cyanuric acid crystals due to the polarizable nature of crystals formed in the kidneys of broilers fed melamine and cyanuric acid combinations. Melamine alone hindered growth performance of broiler chicks and turkey poults but was not significant in most cases until included in the diet at 1.5%. Birds fed cyanuric acid alone did not experience renal failure and had normal growth performance. The presence of melamine in bile may suggest that avian species are capable of clearing melamine from the body via this excretory route, especially considering the concentrations of melamine and cyanuric acid in these studies were significantly higher than that which was inadvertently fed to companion animals. In contrast to research in cats and dogs, the addition of cyanuric acid to melamine contaminated diets alleviated the negative effects that were observed in turkeys fed melamine alone. Additionally, melamine toxicity could be classified as acute toxicity considering the first 10 days of treatment were critical for birds fed melamine, while those that survived the 21 days of treatment had only developed mild renal pathology, and retained full capacity of renal function.

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