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A No Observable Adverse Effects Level (NOAEL) for pigs fed melamine and cyanuric acid

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ABSTRACT

Ingesting melamine adulterated milk products led to kidney stones in many infants in 2008. This differs from the renal failure caused by intratubular crystal formation after co-ingestion of melamine (MEL) and cyanuric acid (CYA) in adulterated pet foods in 2007. To better understand the potential risk of developing crystal nephropathy following co-ingestion of MEL and CYA, we fed 16 weanling pigs 0, 1, 3.3, 10, 33, or 100 mg/kg bw/day of each MEL and CYA, or 200 mg/kg bw/day of either compound individually for 7 days. Crystals were found in the renal medulla and cortex and urine sediments of all pigs fed both MEL and CYA each at 10 mg/kg bw/day (or greater). Crystals were also found in one of the two pigs fed 200 mg/kg bw/day MEL-only. In a 28 day study, 36 weanling pigs were fed 0, 1, or 3.3 mg/kg bw/day of MEL and CYA or 200 mg/kg bw/day MEL-only. Only one of the 3.3 mg/kg MEL and CYA pig kidneys contained crystals. The no-observed-adverse-effect level (NOAEL) for pigs fed MEL and CYA for 28 days was concluded to be 1.0 mg/kg bw/day corresponding to 25 mg/kg (ppm) MEL and 25 mg/kg (ppm) CYA in dry feed.

1. Introduction

In 2007, melamine and a mixture of other triazines including cyanuric acid were intentionally added to food commodities such as wheat gluten and rice protein concentrate to boost apparent protein content. When those ingredients were used to make pet food, many cats and dogs in the US, Canada and South Africa developed kidney failure due to formation of melamine–cyanurate crystalline spherulites in renal tubules (Brown et al., 2007; Cianciolo et al., 2008; Puschner and Reimschuessel, 2011; Reyers, 2007). Retrospective studies have found that episodes of melamine related renal failure (MARF) had occurred previously in Korea, Spain and Thailand but had not been clearly characterized before 2007 (González et al., 2009; Nilubol et al., 2009; Yhee et al., 2009). While melamine and cyanuric acid have a variety of industrial and agricultural uses, neither is an approved additive for human or animal foodstuffs.

Animals destined for human consumption were also exposed to contaminated feed. Adulterated wheat gluten and pet food byproducts contaminated with melamine and related triazines had been used in feeds for hogs, chicken and fish. As a result, 56,000 hogs were placed under quarantine in seven states (USDA, 2007a). These animals were later released for slaughter and human consumption once samples of pork were found to have residue levels of less than 0.05 mg/kg (50 ppb) wet weight melamine, and unlikely to be a human health risk (USDA, 2007b). Subsequent modeling studies based on serum values indicate a withdrawal time for pigs exposed to melamine would likely be less than one day (Buur et al., 2008). Further, melamine's half life in pigs is 4.04 h and therefore residence time in tissues was considered insignificant (Baynes et al., 2008). However neither of these studies investigated the toxicity or persistence of residues following co-ingestion of melamine and cyanuric acid and risk assessments at that time concentrated on exposure to MEL alone (USFDA, 2007).

As the pet food contamination investigations continued, in 2008 hundreds of thousands of Chinese children were affected by

Abbreviations: MEL, melamine; CYA, cyanuric acid; MEL + CYA, melamine and cyanuric acid; NOAEL, No Observable Adverse Effects Level; Mg/kg bw/day, milligrams per kilogram body weight per day; Kg, kilograms; ppm, parts per million; USFDA, United States Food and Drug Administration; CVM, Center for Veterinary Medicine; MD, Maryland; IN, Indiana; ME, Maine; N, sample number; BUN, blood urea nitrogen; CREA, creatinine; NBF, neutral buffered formalin; GLP, good laboratory practices; °C, degrees celsius; Mg/dl, milligram per deciliter; WHO, World Health Organization.

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melamine contaminated infant formula resulting in the death of six children due to renal failure (WHO, 2008). The median level of melamine in infant formula raw materials was 188,000 mg/kg dry weight, whereas comparably trace amounts of cyanuric acid (3.2 mg/kg), ammeline (14.9 mg/kg) and ammelide (293 mg/kg) were detected (WHO, 2008; Wu et al., 2009). The range of melamine concentrations in infant formula samples was <0.05–4700 mg/kg dry weight (WHO, 2008; Wu et al., 2009). In contrast to the intra tubular melamine–cyanuric acid crystals seen in pets, much larger urinary tract stones composed of melamine and uric acid had formed in approximately 300,000 children who drank contaminated infant formula (Sun et al., 2008; Guan et al., 2009; Kuehn, 2009; Shen et al., 2009). In response to this public health crisis, health organizations around the world conducted risk assessments to evaluate risk to consumers, again, focusing on exposure to MEL alone (Anon, 2008; AQSIQ, 2008; Centre for Food Safety, Hong Kong, 2008; Commission of the European Communities, 2008; New Zealand Food Safety Authority, 2008; USFDA, 2008a,b).

Until the events of 2007 and 2008, both MEL and CYA were considered relatively non-toxic compounds. The LD50 for rats has been reported to be 3161 mg/kg bw for MEL and >5000 mg/kg bw for CYA (IUCLID, 2000a,b). Stones and urinary tract epithelial hyperplasia or neoplasms were reported in rats chronically exposed to melamine for 2 years (Ogasawara et al., 1995; Melnick et al., 1984; NTP, 1983). Further, micro-crystals were reported on gross post mortem but no histopathologic lesions were described (Ogasawara et al., 1995). The combined toxicity of MEL- and CYA-only became apparent in 2007 when pets developed renal failure due to the intratubular precipitation of melamine–cyanurate crystals. Subsequent experimental models in which melamine and cyanuric acid were fed at a ratio of 1:1 have clearly demonstrated that ingestion of these compounds together causes crystal formation and subsequent renal failure in cats, fish, pigs and rats (Reimschuessel et al., 2008; Puschner et al., 2007; Chen et al., 2009; Dobson et al., 2008; Kim et al., 2010; Kobayashi et al., 2010; Xie et al., 2010). It is important to note, however, that whereas the pet food adulteration was in close to a 1:1 MEL:CYA ratio, and the milk products were adulterated with almost pure MEL, these were not always the triazine adulterants nor ratios seen in the past, and there is no guarantee that these will be the triazine compounds nor ratios of adulterants in the future (González et al., 2009; Nilubol et al., 2009; Yhee et al., 2009).

During these incidents and risk assessments, knowledge gaps were identified, specifically the lack of dose response data for combined MEL and CYA ingestion. At the time, the USFDA's Center for Veterinary Medicine (CVM) was in the process of conducting a No Observable Adverse Effects Level (NOAEL) study on MEL and CYA combined using fish as a non-mammalian model. The 28-day NOAEL, at which no renal crystals were observed for catfish or trout, was 0.5 mg/kg bw/day of each compound (Reimschuessel et al., 2010). The data from fish represents a worst case scenario since fish excrete compounds more slowly than mammals (Reimschuessel et al., 2005). In an effort to more closely model human risks, FDA deemed that NOAEL studies were warranted using a mammalian species. Two species were chosen, rats which are frequently used as a toxicological model, and pigs. The porcine model was chosen as pigs have an anatomically similar kidney to that of humans, and have traditionally been used in urologic research, including increased intrarenal pressure and ureteral obstructive studies (Sampaio et al., 1998; Dalmose et al., 2000; Rawashdeh et al., 2003; Dissing et al., 2008). The study using rats was conducted by the National Center for Toxicological Research (NCTR) (Jacob et al., 2011) and the pig study was conducted by Center for Veterinary Medicine (CVM). The dosages for both studies were harmonized to maximize the comparative value of those studies. Further, as the milk products adulterated with melamine in China in 2008 were relatively free of other triazines including cyanuric acid, and produced melamine–uric acid stones in children and infants, the FDA was also interested in investigating the pathology resulting from exposure to each compound alone.

The key parameter evaluated in this study was crystal formation observed by examining fresh renal tissue (wet mount sections). Crystal presence in kidneys has been shown to be a more sensitive indicator of effect than routine histopathology or clinical hematology such as blood urea nitrogen (BUN) and creatinine. Crystals have been seen in kidneys of rats given combined MEL and CYA at dosages where the BUN and creatinine remain normal (Jacob et al., 2011). Herein we report the results of a pilot 7 day study and a 28 day exposure study to determine the NOAEL for crystal formation in weanling pigs following co-ingestion of melamine and cyanuric acid, and also to investigate the crystal formation potential of pigs dosed with each compound alone.

2. Methods

2.1. Animals, husbandry and test articles

2.1.1. Seven day range finding study

Weanling cross-bred barrows ($n = 16$, 23 ± 3 kg bw) were obtained from a local producer. Ear-tagged pigs were housed in individual indoor pens at the USFDA's Center for Veterinary Medicine, Office of Research in Laurel, MD and acclimated for at least 2 weeks. A standard health check was performed by the attending veterinarian upon the pigs' arrival to our facility. Pigs were fed a standard corn and soybean diet (18% crude protein) prepared at our facility and had unrestricted access to fresh water. All feeds, including those from the local producer, were tested for the presence of MEL and CYA by liquid chromatography tandem mass spectrometry and no peaks were detected above the limit of quantification, 0.5 mg/kg dry weight (Heller and Nochetto, 2008). Pigs were weighed daily and were observed twice daily for signs of distress or other health issues.

2.1.2. Twenty eight day study

Weanling pigs ($n = 36$, 24 ± 2 kg bw) were also obtained from local farms and housed as stated above for the pilot study.

2.1.3. Test articles

Melamine (MEL; 99% pure; Sigma–Aldrich Co., St. Louis, MO, USA) and/or cyanuric acid (CYA; 98% pure; Sigma–Aldrich Co., St. Louis, MO, USA) were mixed with retail chocolate pudding in amounts tabulated from daily weight measurements to ensure accurate dosing. Pudding also tested negative for melamine and cyanuric acid. All pigs ate all pudding each dosing day and thus received complete test doses.

2.2. Experimental design

2.2.1. Seven day range finding study

In the 7 day study, pigs were randomly assigned to treatment groups of 0, 1.0, 3.3, 10, 33, or 100 mg/kg bw/day of melamine and cyanuric acid each (MEL + CYA) or 200 mg/kg bw/day of either compound individually (MEL or CYA; $n = 2$ each treatment). The notation used in this report is that a dosage of 100 MEL + CYA indicates 100 mg/kg bw/day of MEL and 100 mg/kg bw/day of CYA were fed for a total of 200 mg/kg bw/day of triazines. MEL and CYA were given in a ratio of 1:1 as this was the ratio that had been shown to cause crystals in pigs, fish, cats and rats in previous studies and the doses were chosen to harmonize results with a rat study at the National Center for Toxicological Research (Puschner