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Tissue deposition and residue depletion in rainbow trout following continuous voluntary feeding with various levels of melamine or a blend of melamine and cyanuric acid

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ABSTRACT

This study determined the deposition and depletion in rainbow trout after continuous administration of melamine (MEL) alone or a blend of MEL and cyanuric acid (CYA). The plasma, muscles, kidneys, liver and gills were sampled at 0, 3, 7, 13, 21, 28 and 42d. After the final sampling at 42d, fish from the MEL0.05, MEL20 and MCA groups were fed the control diet (MEL0) for the depletion test. Co-administration with cyanuric acid accelerated the deposition time to the Css for melamine; during the withdrawal phrase, the melamine and CYA concentrations in the tissues decreased exponentially. Compared to the $t_{1/2}$ for single oral administration, the $t_{1/2}$ for melamine and cyanuric acid after 42d continuous feeding was prolonged. The presence of trace CYA in the plasma and kidneys of trout was detected in the MEL20 group, indicating that MEL can convert into CYA in rainbow trout.

1. Introduction

In 2007 and 2008, multiple episodes of melamine (MEL) contamination in pet food and even infant milk products were revealed; thousands of cases of illness and renal failure resulted from the ingestion of contaminated food containing MEL and CYA (Brown et al., 2007; WHO, 2009) and the "stone child" are still suffering from the lithiasis up to now. China is the largest producer and consumer of MEL in the world. MEL and its analogues were intentionally added to protein sources for animal feed because of its high nitrogen content, which artificially raises the crude protein level when using a nitrogen-based test for measuring protein levels.

MEL is an organic base and a trimer of cyanamide with a 1,3,5-triazine skeleton. Like cyanamide, MEL contains 66% nitrogen by mass and if mixed with resins, has fire-retardant properties and several other industrial uses for laminates, plastics, coatings, glues, adhesives and kitchenware. MEL is also a metabolite of cyromazine, which is a pesticide. MEL is formed in mammals that have ingested cyromazine. A study has reported that cyromazine can also be converted into MEL in plants (Lim et al., 1990). Cyanuric acid (CYA, s-triazine-2,4,6-triol) is a type of structural analogue of MEL and is used as a stabiliser in outdoor swimming pools and hot tubs to minimise the decomposition of hypochlorous acid by light (Allen et al., 1982). Therefore, exposure to MEL and related trazine compounds is unavoidable.

Both MEL and CYA have been reported to possess low toxicity, with an oral LD50 in rats of 3161 mg/kg and 7700 mg/kg body mass, respectively (OECD, 1998, 1999), and no human data could be found for the oral toxicity of MEL or CYA separately until the problems with Chinese milk powder. In 2008, the US FDA established a tolerable daily intake (TDI) for MEL of 0.63 mg/kg body weight (bw), and this TDI was subsequently revised to 0.063 mg/kg bw (USFDA, 2007, 2008). In 2009 and 2010, the WHO and EFSA established a MEL TDI of 0.2 mg/kg bw because MEL was found to be much more toxic in the presence of CYA, which was confirmed as the key reason for renal failure in dogs, cats, pigs and fish (WHO, 2009; EFSA, 2010). Considering that animal feed stands at the front of the food chain for humans, the MEL and CYA residues in animal food should not be overlooked. Recently, many studies have been conducted on the deposition and depletion patterns in

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mammals, poultry and fish. In addition to the toxicology, several studies have reported the concentrations of MEL and CYA in swine, poultry, ruminants, fish, shrimp, milk and eggs (Baynes et al., 2010; Karbiwnyk et al., 2010; Lv et al., 2010; Qin et al., 2010; Reimschuessel et al., 2010a; Andersen et al., 2011). Mammals and poultry have been shown to clear MEL much more quickly than fish due to the different metabolic rate (Xue et al., 2011). The $t_{1/2}$ of MEL in the plasma of mammals and broilers was 2.71 to 4.9 h (Mast et al., 1983; Baynes et al., 2008; Yang et al., 2009; Liu et al., 2010) and 3.2 to 6.6 h (Ding et al., 2012), respectively, while the $t_{1/2}$ in trout plasma was 32.2 to 32.9 h (Xue et al., 2011). Thus, fish accumulate MEL to a greater extent than ruminants and poultry (Andersen et al., 2011). Furthermore, MEL administration with or without CYA could affect the elimination of MEL. Reimschuessel et al. (2010a) have reported that the $t_{1/2}$ values for MEL in the muscles of catfish and trout were 1.51 d and 3.62 d, respectively, and were delayed to 1.67 d and 4.04 d after single oral administration if MEL and CYA were given together.

Most of the data mentioned above were mainly obtained from single dose administration in which force-feeding by gavage is commonly used. However, long-term voluntary consumption of contaminated feed is much more likely in practice. Recent studies have disclosed that the oral routes for MEL, including force-feeding by gavage and voluntary feeding, have different effects on renal crystal formation in rats and the $t_{1/2}$ for MEL in rainbow trout (Sprando et al., 2012; Stine et al., 2012). Compared to voluntary feeding, force feeding by gavage has more serious effects on the post-feeding alkaline tide in rainbow trout (Cooper and Wilson, 2008), which is a causative agent of calcium oxalateurinary stones in cats and potentially other species (http://en.wikipedia.org/wiki/Alkaline_tide). Thus, MEL metabolism could be affected by oral routes, and MEL deposition and depletion during voluntary feeding would be different from force feeding by gavage. However, few studies have focused on the influence of chronic voluntary MEL and CYA administration in fish. Without a clear understanding of the deposition and depletion patterns of MEL and CYA in animal tissues, assessing the risks imposed by MEL and CYA contamination and providing the appropriate withdrawal interval in animal products are difficult for the public.

Compared to livestock and poultry, fish require much higher protein content in their diet because of their low carbohydrate utilisation capability (Geurden et al., 2007; Kaushik and Seiliez, 2010). Rainbow trout (*Oncorhynchus mykiss*) is a cold-water carnivorous species that requires a dietary intake of at least 35% protein for normal growth (Wilson, 2002). Many proteins sources, such as fishmeal, meat meal, corn and wheat gluten meal, in China contained MEL in 2009 (Qin et al., 2010) and later years (www.moa.gov.cn). Until now, few studies have been conducted on the renal effects and residue depletion of MEL and CYA in several fish species (Reimschuessel et al., 2008, 2010a,b; Andersen et al., 2011). Our previous study on rainbow trout determined the pharmacokinetic parameters for MEL and a blend of MEL and CYA after a single dose (Xue et al., 2011). The current study was conducted to determine the accumulation and withdrawal of MEL and CYA in the plasma, muscles, kidneys, liver and gills following a low or high level of continuous voluntary oral administration.

2. Materials and methods

2.1. Chemicals

MEL (99%) and CYA (98%) were obtained from Sigma-Aldrich (Shanghai, China). A stable isotope-labelled internal standard was used for LC–MS/MS analysis. [$^{15}N_3$]-MEL and [$^{13}C_3$]-CYA (99% isotopic purity) were supplied by Cambridge Isotope Laboratories, Inc. (Beijing, China). All of the other chemicals and solvents used in the analyses were reagent grade.

2.2. Animals

Rainbow trout (*O. mykiss* Richardson, Salmonidae) were obtained from a farm at the Beijing Fisheries Institute and were transferred into the tanks 2 weeks before acclimatisation. During the acclimatisation period, the fish were fed a commercial diet (440 g/kg crude protein and 160 g/kg crude lipid, produced by Uni-President Inc., Guangdong, China) to visual satiation. Prior to use, all of the feeds and ingredients were tested for MEL and CYA by liquid chromatography-triple quadrupole mass spectrometry (LC–MS/MS) (Qiu et al., 2009). No residues were detected above the limit of detection (LOD) for MEL (0.05 μg/g) or CYA (0.1 μg/g).

The feeding trial was performed in the flowing water facilities of the National Aquafeed Safety Assessment Centre, Feed Research Institute, Chinese Academy of Agricultural Sciences (Beijing, China), using 30 fibreglass cylindro-conical tanks (capacity: 265-L; water-flow rate: 600 mL/min) at a temperature of 15 ± 1 °C. Six tanks with 180 fish were assigned to each treatment to ensure enough fish for sampling. All of the fish with a mean body weight of 124 ± 5 g were randomly allocated to each tank after 24 h of starvation. Dissolved oxygen was maintained above 8 mg/L, the pH was 7.5–8.5, and the ammonia was <0.5 mg/L. The photoperiod was 12L: 12D with a light period from 07:00 to 19:00 h.

2.3. Dosing and sampling

During the feeding period, experimental fish were complied with Laboratory Animal Welfare Guidelines of China (Decree No. 2 of the Ministry of Science and Technology, issued in 1988). Five extrusion diets were prepared with 0, 5, 20 and 2000 mg/kg MEL. Additionally, a diet with a blend of 500 mg/kg MEL and 167 mg/kg CYA was designed. The MEL and CYA blend was designed at 3:1 according to the ratio of scrap MEL in China, which was the main adulteration form for triazines added illegally in the feed (Xue et al., 2011). The formulation and proximate compositions of the trial diets were shown in Table 1. The diets were administered at 1% (80% of ad libitum) of fish body weight each day by half and half at 9:00 am and 4:00 pm with a target MEL dose of 0, 0.05, 0.2 and 20 mg/kg bw and the blend of MEL and CYA at 5 mg/kg bw and 1.67 mg/kg bw, respectively, to each tank for 42 d. The five treatments were named MEL0, MEL0.05, MEL0.2, MEL20

Table 1
Formulation (g/kg), proximate compositions (g/kg) and energy content (MJ/kg) of the experimental diets.

Ingredients	C	MEL0.05	MEL0.2	MEL20	MCA
Peru fishmeal	38	38	38	38	38
Soybean meal	21	21	21	21	21
Squid liver meal	5	5	5	5	5
Fish oil	5	5	5	5	5
Soy oil	5	5	5	5	5
Premix[a]	2.5	2.5	2.5	2.5	2.5
Wheat flour	23.4	23.4	23.4	23.4	23.4
Y_2O_3[b]	0.1	0.1	0.1	0.1	0.1
Melamine (mg/kg)[b]	0	5	20	2000	500
Cyanuric acid (mg/kg)[b]					167
Total	100	100	100	100	100
Analysed Nutrients compositions (in mash, as is, g/kg)					
Moisture	6.33	6.61	6.56	6.37	6.23
Crude protein	42.3	42.73	42.21	43.07	42.6
Crude lipid	13.78	13.92	14.14	14.24	14.12
Ash	8.77	8.66	6.66	8.79	8.85
Gross energy (MJ/kg)	19.92	20.16	20.13	20.13	21.21
Melamine (mg/kg)	/	4.92	20.66	1910.37	491.77
Cyanuric acid (mg/kg)	/	/	/	/	176.40

[a] Vitamin and mineral premix (see Liu et al., 2009).
[b] Test compound and Y_2O_3 were mixed with premix, balanced by zeolite.