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A study on growth, histopathology and oxidative stress in Asian sea bass on diets with various loadings of melamine and cyanuric acid adulterants

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ABSTRACT

Effects of melamine (MEL) and its analogue, cyanuric acid (CYA) at various doses on Asian seabass (*Lates calcarifer* Bloch) are described. Diet 1 (a basal diet without MEL and CYA); diets 2–5 (with MEL and CYA at inclusion levels 2.5, 5, 7.5, 10 g/kg diet, each); diet 6 (with only MEL at 10 g/kg diet); and diet 7 (with CYA alone added at 10 g/kg diet) were examined. It is obvious that those fish that received combined MEL–CYA as follows had low growth and feed conversion ratio (FCR) ($P < 0.05$): 7.5:7.5, 10:10 or MEL alone diets. Abnormalities were observed in the liver and kidney of fish with combined MEL and CYA supplementation. The renal tubules of fish that were fed with diets 2–5 had golden-brown melamine–cyanurate crystals. Fish given only one type of supplementation did not have such crystals in the kidneys. The highest MEL residue in fillet was detected in the fish that ingested MEL alone (10 g/kg diet). Levels of heat shock protein (Hsp) 70 were elevated in the liver of fish that had ingested MEL/CYA, in combination or alone (diets 2–7) ($P < 0.05$). There were no significant differences between the treatments ($P > 0.05$) in the level of Hsp70 in the kidneys of the fish. High dosages of MEL–CYA induced the activities of catalase and glutathione peroxidase in liver and kidneys.

1. Introduction

The toxicity of melamine (MEL) has been reported widely in humans and animals after an outbreak of renal failure in dogs and cats in North America in 2007 (Brown et al., 2007; Cianciolo et al., 2008; Reimschuessel and Puschner, 2010). Since MEL was detected in food and animal feeds, the World Health Organization (WHO) and the U.S. Food and Drug Administration (US-FDA) began assessing the risk and safety levels to comprehend the effects that ingestion of contaminated edible tissues can have on human health (Reimschuessel et al., 2010; US-FDA, 2007; WHO, 2008). Melamine (1,3,5-triazine-2,4,6-triamine) is a nitrogen rich molecule that is used to produce many consumer products, and it has a high about 67% content of nitrogen by molecular weight (Bischoff, 2011). Therefore, the addition of MEL in foodstuff can increase the apparent protein content, as feed protein concentration is traditionally measured by analysis of total nitrogen content (US-FDA, 2007). A toxicity study of MEL in hybrid catfish [*Clarias macrocephalus* (Günther) × *Clarias gariepinus* (Burchell)] showed the adverse effects of this toxic substance on growth and blood components, and histological alterations (Janlek et al., 2009). In addition, MEL also presented negative effects on growth and dorsal skin melanin content in dark-barbel catfish, *Pelteobagrus vachelli* (Richardson) (Xue et al., 2011). Phromkunthong et al. (2013) found that red tilapia ingesting MEL at high dosages had poor growth performance and feed efficiency. Furthermore, histopathological alterations were detected in the kidney, liver and gills of the melamine treated fish. The accumulation of MEL residues in fish reflected its dietary levels.

An incident of melamine and cyanuric acid (CYA) contamination has been found in feed ingredients (Karbiwnyk et al., 2012; Stine et al., 2012). Data on toxicity of this combination in aquatic animals are particularly scarce. Even though MEL and CYA at low concentrations are known to be relatively nontoxic when taken individually, some recent research indicates the potential of toxic interaction for the MEL and CYA combination (Pirarat et al., 2012; Puschner et al., 2007; Reimschuessel et al., 2008). Several studies of renal failure reported in pets and farm animals, including aquatic species and ruminants that received MEL and CYA at high dosages, found crystals in the kidneys, similar to the pathological signs of renal failure (Brown et al., 2007; Nilubol et al., 2009; Puschner et al., 2007; Reimschuessel et al., 2008; Sun et al., 2010; Thompson et al., 2008). To understand the effects of MEL and its derivative (CYA) more clearly, Phromkunthong et al. (2014) studied the effects of MEL or CYA and MEL–CYA combination on tilapia, through histopathological observations and using stress biomarkers. They also found that the combination of MEL and CYA induces crystal nephropathy. The purpose of our current study was to assess the toxicity of MEL and CYA alone and in combination when fed to Asian sea bass, to describe its effects on pathophysiological changes or stress biomarkers,

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and to evaluate the bioaccumulation of the toxic chemicals in edible tissues to assess the safety of Asian sea bass as food. Because the protein content of animal feed affects its price, there is incentive to add MEL or CYA to the feed. However, singly or in combination these could either harm the animals, or become a food safety problem. The food safety risk is highest with animals that are farmed and significantly consumed as food. Each such species individually provides a pathway and poses a risk, with varying characteristics in animal health effects and in transmission to humans. For food safety, these different pathways should be individually assessed. Ideally we would like to find a species that will not transmit, either because it is sensitive to the contamination, or because the profile of accumulation prevents it. Alternatively, we need to know if some species are a particularly high food safety risk. This paper is the second in a series that assesses such pathways in the aquaculture of Thailand. The first one assessed tilapia, while this one focuses on sea bass. The results show significant differences between these two species in their transmission characteristics.

2. Materials and methods

2.1. Experimental set-up and fish

Asian sea bass, *Lates calcarifer* Bloch, fingerlings of an average weight of 4.77 ± 0.25 g (mean ± SD) were obtained from the National Institute of Coastal Aquaculture (NICA), Songkhla, Thailand. After confirming that they were free of pathogens, they were moved to the facilities of Kidchakan Supamattaya Aquatic Animal Health Research Center, Department of Aquatic Science, Faculty of Natural Resources, Prince of Songkla University, Songkhla, Thailand. The fish were acclimatized for one month to a MEL/CYA non-supplemented diet (diet 1, see Table 1) in a 1000 L fiberglass tank containing dechlorinated freshwater. At the start of the experiment, fish with an average body weight 8.62 ± 0.01 g (mean ± SD, $n = 504$) were randomly transferred to 28 glass aquaria (184 L; $n = 18$ fish per aquarium). Following this, the fish were fed three times a day to satiation at 8:00, 12:00 and 16:00 h. Feces and uneaten feed were removed to maintain water quality every day. Over the 12-week rearing period the water temperature varied within 27–28 °C, pH within 7.5–7.7, and dissolved oxygen was not less than 6.0 mg/L. The mean values for total ammonia and nitrite were 0.1–0.2 mg/L and 0.01–0.03 mg/L, respectively. The water quality parameters mentioned here were within the acceptable ranges reported for sea bass (Thai Agricultural Commodity and Food Standard TACFS 7412-2007, 2007).

2.2. Preparation of diets

MEL with a purity better than 99.5% was supplied by Chang Chun Petrochemical Co. Ltd., Taiwan. CYA (98% purity) was obtained from Sigma-Aldrich (St Louis, MO, USA). Experimental diets were formulated with fishmeal as the major protein source. Furthermore, soybean meal, shrimp head meal, wheat flour, rice flour and wheat gluten were used as ingredients of the control diet (diet 1) providing 400 g/kg diet nitrogen content and about 120 g/kg diet crude lipid (Table 1). The feeds were made into sinking pellets (pellet diameter 3 mm) using a Hobart mixer (Model A200T, USA), oven-dried at 60 °C and stored at −20 °C. The details of feed preparation are described in our prior study (Phromkunthong et al., 2013). The seven experimental diets were as follows: control diet (without supplementation of MEL or CYA, diet 1), and in the other diets the MEL:CYA supplementations (in g/kg feed) were 2.5:2.5 (diet 2), 5:5 (diet 3), 7.5:7.5 (diet 4), 10:10 (diet 5), 10:0 (diet 6) and 0:10 (diet 7). Three feed samples from each treatment group were analyzed for their proximate chemical compositions, using the procedures of AOAC (1995) (Table 1). Dry matter was determined by drying the samples at 105 °C until constant weight. Nitrogen content ($N \times 6.25$) was determined by Kjeldahl method using Kjeltec protein analyzer (Kjeltec™ 8100, FOSS, Tecator, Sweden). Crude lipid was determined by methylene chloride extraction using Soxhlet method (Soxtec System HT1043, Sweden). Ash was measured after combustion in a muffle furnace (Gallenkamp Box Furnace, UK) at 550 °C for 6 h. The concentrations of MEL and CYA in the diets were determined by LC-MS/MS (Central Laboratory (Thailand) Co. Ltd., Bangkok, Thailand) using an adaptation of the method US-FDA LIB No. 4422 with HILIC chromatography (Smoker and Krynitsky, 2008), as described in our previous publication (Phromkunthong et al., 2013).

Table 1
Formulations and proximate compositions of the experimental diets.

Ingredient composition (g/kg diet)	Diets (MEL:CYA, g/kg diet)						
	Diet 1 (control)	Diet 2 (2.5:2.5)	Diet 3 (5:5)	Diet 4 (7.5:7.5)	Diet 5 (10:10)	Diet 6 (10:0)	Diet 7 (0:10)
Fishmeal	380	380	380	380	380	380	380
Soybean de-hulled	110	110	110	110	110	110	110
Shrimp head meal	100	100	100	100	100	100	100
Wheat flour	190	190	190	190	190	190	190
Rice flour	89.8	89.8	89.8	89.8	89.8	89.8	89.8
Wheat gluten	37	37	37	37	37	37	37
Vitamin & mineral premix[a]	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Fish oil:soybean oil (1:1)	60	60	60	60	60	60	60
Mono-calcium phosphate	10	10	10	10	10	10	10
Choline chloride	1	1	1	1	1	1	1
Inositol	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Antioxidant (BHT)	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Microcrystalline cellulose	20	15	10	5	0	10	10
Melamine (MEL)[b]	0	2.5	5	7.5	10	10	0
Cyanuric acid (CYA)[c]	0	2.5	5	7.5	10	0	10
Proximate composition (g/kg as dry matter basis, analyses of three batches of diet given as mean ± SD)							
Nitrogen content (N × 6.25)	40.78 ± 0.10	43.42 ± 0.21	44.20 ± 0.10	44.94 ± 0.10	47.26 ± 0.44	44.66 ± 0.31	43.53 ± 0.03
Lipid	12.06 ± 0.11	12.26 ± 0.60	11.51 ± 0.10	10.93 ± 0.01	11.81 ± 0.11	10.78 ± 0.22	11.67 ± 0.02
Ash	12.88 ± 0.26	13.31 ± 0.19	13.81 ± 0.56	13.07 ± 0.38	12.22 ± 0.05	13.12 ± 0.06	13.49 ± 0.25
Gross energy (MJ/kg diet)	16.37	16.37	16.37	16.37	16.37	16.37	16.37

[a] Vitamin & mineral premix deliver the following in unit kg^{-1} diet: retinal (A) 8000 IU; cholecalciferol (D3) 1500 IU; tocopherol (E) 100 mg; menadione sodium bisulfite (K3) 5 mg; thiamine (B1) 10 mg; riboflavin (B2) 15 mg; pyridoxine (B6) 15 mg; cobalamin (B12) 0.02 mg; niacin 80 mg; calcium pantothenate 40 mg; ascorbic acid (C) 150 mg; biotin 0.5 mg; folic acid 4 mg; Cu 5 mg; Fe 30 mg; Zn 40 mg; Mn 25 mg; Co 0.05 mg; I 1 mg; Se 0.25 mg.
[b] Melamine: Chang Chun Petrochemical Co., Ltd., Taipei Taiwan (purity 99.5%).
[c] Cyanuric acid (98%): Sigma, Aldrich Co., Ltd.