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Residue Depletion of Melamine in Pigs Exposed to Melamine Contaminated Feed

Mu Li, Limin Gong, Hui Wu, Liying Zhang, Binru Shang and Yiqiang Chen
State Key Laboratory of Animal nutrition, China Agricultural University,
100193 Beijing, Poeple's Republic of China

Abstract: The residue depletion profile of melamine in pigs exposed to melamine contaminated feed was studied. A total of 56 Landrace x Hampshire x Duroc crossbred pigs weighing 30.6±1.8 kg were blocked by weight and randomly assigned to one of two treatments with ratio of barrow to gilt 1:1 and fed diets supplemented with 30 and 100 mg kg^{-1} melamine for 42 days. The pigs were housed in pens in groups of seven and each treatment was fed to four pens. One pig from each replicate was slaughtered at 0, 12, 24, 48, 96 and 120 h after withdrawing the melamine contaminated diets and samples of kidney, *Longissimus dorsi* muscle, plasma and urine were analysed for melamine by liquid chromatography-tandem mass spectrometry. The results indicated that the melamine content was higher in the kidney than that in *Longissimus dorsi* muscle and plasma. The melamine concentrations in urine were much higher than in tissues and plasma. The depletion of melamine residue in pig tissues is relative rapid, its content in kidney, *Longissimus dorsi* muscle and plasma at 12 and 24 h after withdrawing melamine contaminated diets decreased by around 50 and 70%, respectively. If a melamine withdraw interval was estimated based on pig kidney residues, it will be 96 h and over 120 h for pigs exposed to 30 and 100 mg kg^{-1} melamine contaminated diets for 42 days, respectively.

Key words: Pigs, melamine, depletion, tissues, plasma, withdrawal interval

INTRODUCTION

Melamine (1, 3, 5-triazine-2, 4, 6-triamine; Fig. 1) is a triazine-based chemical used in the manufacture of plastics and flame retardants. Although, it is not approved for use as a feed or food additive in any country, it has been illegally added into feedstuffs or milk to increase nitrogen amount without addition of protein. Now melamine is a well known feed and food adulterant and contaminant worldwide. Its occurrences in the ingredients of pet foods imported from China that resulted in killings of pets and its involvement in the toxic milk that caused kidney failure and deaths in infants in China have made melamine a focus of international food safety concern in recent years (Hsieh *et al*., 2009).

Melamine itself is a low toxic chemical with an oral LD_{50} of 3160 mg kg^{-1} in the rats, it can bind with its analogues such as cyanuric acid to form crystals and then may induce significant renal toxicity and carcinogenic effects (OECD, 1998). Therefore, Tolerable Daily Intake (TDI) 0.063 and 0.2 mg kg^{-1} body weight per day have been set by U.S FDA (2008) and WHO (2008), respectively. It was found that most of the oral dose melamine was eliminated in the urine of male Fisher rats within 24 h and there was more melamine recovered in kidney of rats than in other tissues (Mast *et al*., 1983). To establish an appropriate withdrawal internal Buur *et al*. (2008) developed a physiologically based pharmacokinetic model for melamine with rats and it was extrapolated to pigs and applied pig model to estimate the withdraw interval (based on kidney residues) for single oral exposures of melamine, respectively and withdraw interval of chronic oral dosing was estimated. Lastly, Lv *et al*. (2010) reported a withdraw interval of melamine for lambs which were exposed to 100 mg kg^{-1} melamine contaminated diets for 60 days. In this study, the objective was to investigate the residue depletion profile of melamine in pigs after chronic exposed to melamine contaminated feed.

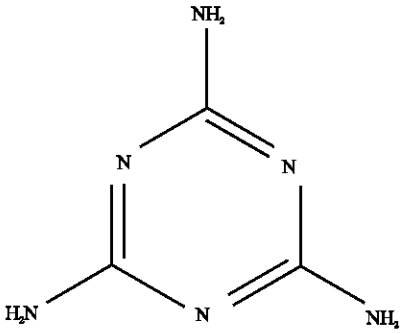

Fig. 1: Chemical structure of melamine

Corresponding Author: Liying Zhang, State Key Laboratory of Animal nutrition, China Agricultural University,
100193 Beijing, Poeple's Republic of China

MATERIALS AND METHODS

Chemicals: The melamine (99.5%) was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). LC-grade acetonitrile (MeCN) and methanol were obtained from Fisher Scientific (Fair Lawn, NJ). Internal standard $^{15}N_3$-melamine (98% chemical purity, 98% isotopic purity) was purchased from Toronto Research Chemicals Inc. (North York, Canada).

Water for analysis was Milli-Q filtered (Millipore, Bedford, MA). All other chemicals were of analytical grade from Beijing Chemical Reagent Corp (Beijing, China).

Animals: The residue study was approved by the Animal Welfare Committee of China Agricultural University. A total of 56 Landrace x Hampshire x Duroc crossbred pigs weighing 30.6±1.8 kg were blocked by weight and randomly assigned to one of two treatments with ratio of barrow to gilt 1:1 and fed diets supplemented with 30 and 100 mg kg^{-1} melamine for 42 days. The composition and nutrient levels of the basal diet are shown in Table 1. The pigs were housed in concrete-floored pens (5.0×7.0 m) in groups of seven and each treatment was fed to four pens of pigs. The pens were equipped with a two-hole feeder and nipple water that allowed *ad libitum* access to feed and water.

Animal treatment and sampling: One pig from each replicate was slaughtered at 0, 12, 24, 48, 96 and 120 h after withdrawing the melamine contaminated diets. Urine samples were obtained prior to slaughter. The pigs were euthanized by cardiac puncture, bled and then the carcass was split along the midline. The abdominal cavity was opened and the liver and kidney were removed. Samples of *Longissimus dorsi* muscle were taken from the right side of the carcass between the 5th and last rib. Tall samples (kidney, *Longissimus dorsi* muscle, plasma and urine) were deep frozen at -75°C until needed for residue analysis.

Standards: Standard stock solution of melamine was prepared by dissolving 100 mg powder, accurately weighed in 100 mL MeCN/H_2O (8:2, v/v, 0.1% formic acid) obtaining a final concentration of 1 mg mL^{-1}. The $^{15}N_3$-melamine stock solutions of 1 mg mL^{-1} were prepared by dissolving 10 mg in 10 mL MeCN/H_2O (8:2, v/v, 0.1% formic acid). The stock solutions were diluted with appropriate volumes of MeCN/H_2O (8:2, v/v, 0.1% formic acid) to prepare working and fortification solutions.

Melamine analysis: An API 2000 Tandem Mass-Spectrometer (AB Sciex Instruments, Foster City, CA) equipped with TurboIonSpray and Agilent 1200 HPLC was used to perform the melamine analysis according to the procedures outlined by Qiu *et al*. (2009).

Table 1: Composition and nutrient levels of the experimental diets (percentage of as-fed basis)

Ingredient (%)	Content	Nutrient (%)	Level[a]
Maize	67.0	Digestible energy (MJ kg^{-1})	14.2
Wheat bran	3.0	Crude protein	18.0
Soybean meal	26.7	Calcium	0.8
Dicalcium phosphate	1.3	Phosphorus	0.6
Limestone	0.9	Lysine	1.0
Vitamin-mineral premix[b]	1.0	Methionine and cysteine	0.6
Lysine HCl	0.1		

[a]Level: the nutrient levels were analyzed values except digestible energy; [b]Premix provided per kilogram of complete diet: vitamin A, 7,500 IU, vitamin D_3, 1,000 IU; vitamin E, 10 mg; vitamin K_3, 2.5 mg; thiamine, 1.5 mg; riboflavin, 5.0 mg; pantothenic acid, 20 mg; niacin, 30 mg; pyridoxine, 4 mg, biotin, 0.5 mg; vitamin B_{12}, 0.05 mg; folic acid, 2.0 mg; choline, 500 mg; manganese, 80 mg; zinc, 110 mg; ferrum, 100 mg; copper, 10 mg; selenium, 0.3 mg; iodine, 0.35 mg

Tissues (kidney, liver, *Longissimus dorsi* muscle and *Biceps femoris* muscle) were minced then homogenized for 2 min. Two grams of homogenate were accurately weighed into a 50 mL polypropylene centrifuge tube fortified with an appropriate volume of 10 μg mL^{-1} $^{15}N_3$-melamine, then 10 mL of acetonitrile/H_2O (7:3, v/v) was added to the samples. After 20 min of vortexing followed by centrifugation at 1.902×g for 10 min, 3 mL of the supernatant were transferred to a 10 mL polypropylene centrifuge tube and 2 mL of hexane were added. The supernatant was vortexed for 1 min followed by centrifugation at 2.219×g for 2 min and the hexane layer was discarded. To 0.5 mL of the lower layer, 0.5 mL acetonitrile were added. After the sample was vortexed and centrifuged at 18.894×g for 10 min, the supernatant was filtered through a 0.22 μm nylon filter before analysis. For plasma samples, 0.5 mL plasma were pipetted into a 50 mL polypropylene centrifuge tube fortified with an appropriate volume of 10 μg mL^{-1}, $^{15}N_3$-melamine and 0.5 mL of H_2O, 7.0 mL of formic acid/H_2O (2/98, v/v) and 2 mL of acetonitrile were added. The solution was vortexed for 30 min followed by centrifugation at 9.391×g for 10 min and then 0.5 mL of supernatant was transferred to a polypropylene centrifuge tube and 1 mL of acetonitrile was added. After centrifugation at 11.180×g for 10 min, the supernatant was filtered through a 0.22 μm nylon filter before analysis.

For urine, 1.0 mL of sample and 4 mL of acetonitrile were pipetted into a polypropylene centrifuge tube fortified with an appropriate volume of 10 μg mL^{-1}, $^{15}N_3$-melamine and centrifuged at 1.753×g for 5 min. Then, 0.5 mL of supernatant were transferred to a centrifuge tube and the solution was diluted to 1 mL with acetonitrile. The sample was centrifuged at 11.180×g for 10 min and the supernatant was filtered through a 0.22 μm nylon filter before analysis.

Statistical analysis: The data of melamine content in tissues, plasma and urine was analyzed using MEANS procedure of SAS (2002).

RESULTS AND DISCUSSION

Method validity: The linearity, accuracy and precision of melamine determination method used in this study were evaluated before application. The melamine standard calibration curve was linear over the concentration range of 2-500 ng mL^{-1} and R^2>0.99. The Limit of Detection (LOD) and the Limit of Quantification (LOQ) were defined on the basis of Signal-to-Noise ratios (S/N) of 3:1 and 10:1, respectively. The LOD and LOQ were 0.02 and 0.05 μg mL^{-1} for plasma, 0.01 and 0.02 μg mL^{-1} for urine, 0.02 and 0.05 μg g^{-1} for tissues. The accuracy of the method was assessed by using blank pig tissues spiked with both melamine at levels of 0.05, 0.2, 2 μg g^{-1}. Recoveries of all fortification levels were from 83-108% for melamine (relative standard deviation, RSD<11%).

Residue depletion: The melamine concentration at 0, 12, 24, 48, 96 and 120 h after withdrawing melamine contaminated diets is shown in Table 2. The results indicated that the melamine content was higher in the kidney than that in *Longissimus dorsi* muscle and plasma. The melamine concentrations in urine were much higher than in tissues and plasma, suggesting that melamine is mainly excreted in the urine by pigs. These results confirmed the assertion by Baynes *et al.* (2008) that the melamine was excreted in the kidney and proved the conclusion there was more melamine recovered in kidney of rats than in other tissues by Mast *et al.* (1983). In this study, it was also showed that the melamine content in tissues, plasma and urine were dose-dependent with melamine contaminated level in diets exposed.

As is shown in Table 3, the content of melamine in kidney, *Longissimus dorsi* muscle and plasma at 12 and 24 h after withdrawing melamine contaminated diets decreased by around 50 and 70%, respectively. This indicates that the depletion of melamine residue in pig tissues is relative rapid (Fig. 2) and this is in accordance with that most of the oral dose melamine was eliminated in the urine of male Fisher rats within 24 h reported by Mast *et al.* (1983). The results also showed that the content of melamine in *Longissimus dorsi* muscle and plasma fell below LOQ at 48 h for 30 mg kg^{-1} melamine treatment but only in *Longissimus dorsi* muscle declined below LOQ at 120 h for 100 mg kg^{-1} melamine treatment. If a melamine withdraw interval was estimated based on pig kidney residues, it will be 96 h and over 120 h for pigs exposed to 30 and 100 mg kg^{-1} melamine contaminated diets for 42 days, respectively.

Comparing with the result recently reported by Lv *et al.* (2010) that it took 96 h for melamine residue in lamb tissues to fall below 0.02 μg g^{-1} which lambs were exposed to 100 mg kg^{-1} melamine contaminated diets for 60 days suggesting that the depletion of melamine was

Table 2: Average melamine residue concentrations in tissues, plasma and urine of pigs after withdrawing melamine from 30 mg kg^{-1} melamine treatment (mean±SD, n = 4)

	Kidney (μg g^{-1})		*Longissimus dorsi* (μg g^{-1})		Plasma (μg mL^{-1})		Urine (μg mL^{-1})	
Withdrawal time (h)	30 mg kg^{-1}	100 mg kg^{-1}	30 mg kg^{-1}	100 mg kg^{-1}	30 mg kg^{-1}	100 mg kg^{-1}	30 mg kg^{-1}	100 mg kg^{-1}
0	0.53±0.10	1.91±0.06	0.25±0.04	0.98±0.16	0.22±0.03	0.87±0.09	28.35±12.12	66.25±14.21
12	0.26±0.09	0.96±0.36	0.14±0.05	0.45±0.10	0.16±0.04	0.44±0.10	24.49±8.680	65.43±10.30
24	0.15±0.04	0.26±0.10	0.08±0.02	0.13±0.08	0.08±0.02	0.12±0.05	11.21±3.450	25.94±8.790
48	0.07±0.02	0.18±0.08	<LOQ[a]	0.09±0.05	<LOQ	0.09±0.04	5.88±1.620	12.95±3.900
72	0.07±0.03	0.13±0.09	<LOQ	0.07±0.03	<LOQ	0.07±0.05	3.82±1.210	8.58±3.060
96	<LOQ	0.12±0.04	<LOD[b]	<LOQ	<LOQ	0.07±0.03	2.24±0.310	2.35±0.820
120	<LOQ	0.10±0.05	<LOD	0.07±0.04	<LOQ	0.06±0.03	0.61±0.280	2.20±0.980

[a]LOQ: Limit Of Quantification; [b]LOD: Limit Of Detection

Table 3: The percentage of melamine depleted from tissues, plasma and urine of pigs

Withdrawal time (h)	Treatment[a] (mg kg^{-1})	Kidney (%)	*Longissimus dorsi* (%)	Plasma (%)	Urine (%)
12	30	50.94	44.00	27.27	13.62
	100	49.74	54.08	49.43	1.24
24	30	71.70	68.00	63.64	60.46
	100	86.39	86.73	86.21	60.85
48	30	86.79	-[b]	-	79.26
	100	90.58	90.82	89.66	80.45
72	30	86.79	-	-	86.53
	100	93.19	92.86	91.95	87.05
96	30	-	-	-	92.10
	100	93.72	92.86	91.95	96.45
120	30	-	-	-	97.85
	100	94.76	-	93.10	96.68

[a]Treatment: The melamine contaminated levels in diet; [b]Melamine content fell below the Limit of Quantification (LOQ)

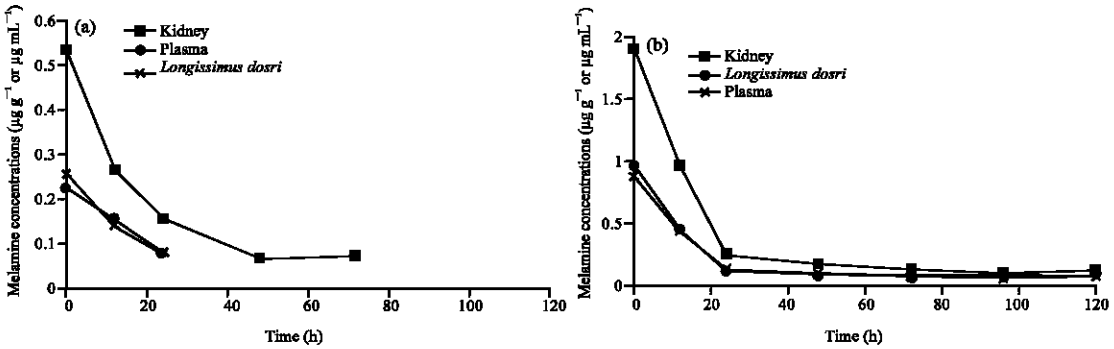

Fig. 2: Depletion of melamine in kidney, *Longissimus dorsi* and plasma of pigs after withdrawing melamine contaminated diets. (a) the 30 mg kg^{-1} melamine treatment and (b) the 100 mg kg^{-1} melamine treatment

slower in pigs than in lambs. Otherwise a physiologically based pharmacokinetic model for melamine with rats was established and extrapolated to pigs by Buur *et al.* (2008) and they applied the pig model to estimate a withdraw interval (based on kidney residues) of 19.2 and 20.9 h for single oral exposures of 3.0 and 5.12 mg kg^{-1} melamine, respectively and a withdraw interval of 20 and 21.3 h for chronic oral dosing (3.0 and 5.12 mg kg^{-1} twice daily for 7 days).

Cruywagen *et al.* (2009) found that the melamine concentration dropped rapidly after changing all cows back to the control dietary and melamine declined to undetectable levels in the milk >6 day after last ingestion of melamine.

These results indicated that the withdrawal interval of melamine in animal tissues is affected by animal species, exposure levels and ways.

CONCLUSION

The results of this study indicated that the melamine content was higher in the kidney than that in *Longissimus dorsi* muscle and plasma. The melamine concentrations in urine were much higher than in tissues and plasma suggesting that melamine is mainly excreted in the urine by pigs.

The depletion of melamine residue in pig tissues is relative rapid, its content in kidney, *longissimus dorsi* muscle and plasma at 12 and 24 h after withdrawing melamine contaminated diets decreased by around 50 and 70%, respectively.

The withdrawal interval of melamine in animal pig tissues is affected by exposure levels and ways. If a melamine withdraw interval was estimated based on pig kidney residues, it will be 96 h and over 120 h for pigs exposed to 30 and 100 mg kg^{-1} melamine contaminated diets for 42 days, respectively.

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