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Full Length Research Paper

Effects of different doses of melamine in the diet on melamine concentrations in milk, plasma, rumen fluid, urine and feces in lactating dairy cows

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The objectives of this paper were to evaluate the effects of feeding diets containing different levels of melamine on melamine concentrations in milk, plasma, rumen fluid, urine and feces in Holstein dairy cows. Sixteen Chinese Holstein dairy cows fixed with permanent ruminal cannulas were assigned to 1 of 4 treatments within a completely randomized design for 10 days. Cows were fed different amounts of melamine {20 (group 1), 40 (group 2), 60 (group 3) or 80 (group 4) g/day/cow} once daily in the morning mixed with a melamine free basal diet for 7-days adaptation followed by 3-days urine and feces sample collections. Melamine was found in all samples tested and its concentration generally increased as dose increased in the diet. These results indicated that different doses of melamine in the diet could result in different concentrations of melamine in milk, plasma, rumen fluid, urine and feces. Data suggested that melamine primarily cleared by urinary excretion, followed by fecal excretion in lactating dairy cows. Mammary tissue was apparently not a major tissue to dispose melamine, especially when fed a relatively low dose (lower than 40 g/day/cow).

Key words: Melamine, excretion, lactating dairy cow.

INTRODUCTION

The fact that melamine appeared in pet food and milk products (World Health Organization, 2008) have induced a widespread food safety consideration. Melamine (2, 4, 6-triamino-s-triazine) is a white crystalline substance first synthesized in the 1830s and commonly used industrially in the manufacture of plastics, laminates, and in some fertilizers. A driving force for the adulteration of feedstuff and milk products with melamine is that its high nitrogen content (66% nitrogen by mass) increases the apparent protein content detected by standard protein analysis tests, for instance, Kjeldahl or Dumas, which consider the total nitrogen level as the indicator of the protein content. Back in the 1960's and 1970's, melamine was investigated as a nonprotein nitrogen (NPN) source for ruminant feed supplementation, but its use in feed has been discontinued due to its incomplete hydrolysis, and it has been found to cause death of experimental animals associated with kidney failure (Clark, 1966; MacKenzie, 1966; Newton and Utley, 1978). In MacKenzie's (1966), study at doses of approximately 250 mg/kg in the diet, sheep refused feed and then lost weight; when fed low-quality hay, some died. Clark (1966) conducted a dose-response study, and found that doses of 25 to 100 g killed sheep, and necropsies revealed tubular damage and kidneys packed with melamine crystals. Newton and Utley (1978) reported that the dose of 45 g in the diet induced four of the six steers to refuse feed.

The maximum residue limits of melamine in foods have been introduced in many countries since the major pet food recall in 2007 and melamine adulteration in infant formula in 2008. The FDA's safety/risk assessment (2007) concluded that a 63 mg of melamine/kg dose in diet is safe. The contaminated pet food was also incorporated into the food supply of several different food animal species. Melamine contamination was determined to a level of 30 to 120 ppm in swine feed (US-FDA, 2007). The public is concerned about consuming milk from cows exposed to melamine. A recent report from South Africa

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Table 1. Ingredients and chemical composition of the basal diet.

Item	Amount (% of DM)
Ingredient	
Corn	30
Soybean meal	7.5
Wheat barn	5
Rapeseed meal (sol.)	1
Cottonseed meal (sol.)	5
Premix[1]	0.5
$CaHPO_4$	0.1
Limestone	0.4
Salt	0.5
Alfalfa	25
Reed	25
Chemical composition	
CP (%)	14.9
NDF (%)	34.1
ADF (%)	20.6
Ca (%)	0.67
P (%)	0.36
NE_L (MJ/kg)	5.77

[1]Contained 1,000,000 IU/kg of vitamin A, 65,000 IU/kg of vitamin D, 5,000 IU/kg of vitamin E, 2,000 mg/kg of Fe, 2,550 mg/kg of Mn, 5,500 mg/kg of Zn, 1,750 mg/kg of Cu, 70 mg/kg of I, 40 mg/kg of Co and 75 mg/kg of Se.

suggested that melamine-contaminated raw materials in dairy cow's feed may originally induce the presence of melamine in cow's milk. In their study, an equivalence dose of 17.1 g melamine feeding lead to a maximum melamine concentration of 15.7 mg/kg in milk (Cruywagen et al., 2009). However, more dose response studies on animal feeding are needed to have a better understanding of the clearance mechanisms of melamine in the animal. The objectives of the present study were to investigate the effect of different levels of melamine in the diet on melamine concentrations in milk, plasma, rumen fluid, urine and feces in Holstein dairy cows.

MATERIALS AND METHODS

Cows, diets and experimental design

All procedures involving the use of animals were approved by the State Key Laboratory of Animal Nutrition in China Agricultural University. Sixteen ruminally cannulated lactating Chinese Holstein cows {195 ± 13 (mean ± SE) in milk; weighing 612 ± 6.2 (mean ± SE) kg} were randomly assigned to one of the four treatments {20 (group1), 40 (group 2), 60 (group 3), or 80 (group 4) g/day/cow of melamine (1,3,5-triazine-2,4,6-triamine, CAS 108-78-1), respectively} in a completely randomized design for 10 days with 7days for adaptation and 3 days for sample collections. The determination of treatment doses of melamine was based on calculations from MacKenzie's study (1966), which suggested that ruminant animals should be able to tolerate dose of 0.16 g melamine/kg BW without any apparent illness. The cows were weighed for three consecutive days at the beginning of the experiment. The lightest cow used in the present study weighed 574 kg; therefore, intake of 20 to 80 g of melamine per cow should not harmfully affect the health of the experimental animals. Cows were maintained in individual tie stalls with free access to fresh water in China Agricultural University Dairy Research Center and fed melamine-free basal diet (Table 1) at equal amounts twice daily at 06:00 and 18:00 h. At each time, cows received 6 kg of concentrate, 3 kg of alfalfa and 3 kg of reed with concentrate and forage delivered separately. Diets were formulated so that the requirements of metabolizable protein, vitamins and minerals were met or exceeded for lactating dairy cows (National Research Council, 2001). At the onset of the trial, pure melamine (20, 40, 60 or 80 g/day/cow, respectively) was mixed with concentrate only in the morning meal and delivered to cows before forage feeding. Orts were collected once per day before the evening feeding. Amounts of feed offered and refused were measured on a daily basis. All cows were milked three times per day at 06:00, 12:00, and 18:00 h throughout the experiment.

Sample collections and preparations

Feed, blood, rumen fluid and milk samples from all cows were collected and analyzed for melamine prior to the beginning of the experiment and no melamine was detected in any of the samples.

During the sample collection period, milk was sampled three times per day after each milking time (0, 6 and 12 h after melamine ingestion), and rumen fluid was sampled 5 times per day (0, 1, 2, 4 and 9 h after melamine ingestion). Milk samples were preserved

Table 2. Effects of different doses of melamine in the diet on melamine concentrations in milk, plasma, rumen fluid, urine and feces in lactating dairy cows.

Item	Treatment[1]				
	Group 1	Group 2	Group 3	Group 4	SEM[2]
Milk (μg/ml)					
0 h	3.54[a]	4.93[a]	7.49[b]	20.63[c]	1.77
6 h	4.78[a]	4.94[a]	10.42[b]	24.53[c]	3.01
12 h	4.39[a]	5.24[ab]	11.29[b]	28.10[c]	2.14
Plasma (μg/ml)					
0 h	0.79[a]	5.64[b]	6.90[b]	15.10[c]	0.96
6 h	3.25[a]	8.05[b]	18.35[c]	23.17[c]	1.73
12 h	0.96[a]	7.80[b]	9.28[b]	20.25[c]	1.55
Rumen fluid (μg/ml)					
0 h	5.76[a]	9.37[a]	17.36[b]	34.10[c]	2.71
1 h	18.32[a]	23.62[ab]	51.11[b]	110.9[c]	9.22
2 h	13.94[a]	17.12[a]	56.04[b]	86.19[c]	10.9
4 h	11.65[a]	15.14[a]	44.36[b]	76.21[c]	9.90
9 h	8.01[a]	13.97[a]	35.49[b]	74.65[c]	4.12
Urine (μg/ml)					
Mean	73.39[a]	100.57[a]	109.05[a]	197.28[b]	24.41
Feces (μg/g)					
Mean	21.38[a]	41.46[b]	47.75[b]	62.38[c]	4.53

[a-c] Means within a row with different superscripts differ ($P < 0.05$). [1]Treatments: group 1 = 20 g melamine/day; group 2 = 40 g melamine/day; group 3 = 60 g melamine/day; group 4 = 80 g melamine/day. [2]SEM = standard error of the mean.

with potassium dichromate and stored at -20℃ until analyzed. Rumen fluid samples were centrifuged at 3000 rpm for 10 min, and supernatant liquid was preserved with 50% sulfuric acid and stored at -20℃ for later analysis. Blood samples were only taken at the last sample collection day to minimize the stress on cows. Peripheral blood (20 ml) was collected at approximately the same time each day (0, 6 and 12 h after melamine ingestion) from the jugular vein into evacuated tubes containing heparin sodium from each cow and centrifuged at 3000 rpm for 15 min and plasma was stored at -20℃ for later analysis.

A total urine collection method for female cattle was adopted from Deliberto and Urness (1995)'s study. The total urine collection apparatus was constructed with a burlap strip, a urinary drainage bag glued with plastic tubing and a liquid collection bucket. The urinary drainage bag glued with a plastic tubing was attached to each cow's vulva and then out around the tuber ischiadica (example, the pins) by hypoallergenic biological adhesive and a burlap strip before the first day of adaptation so that cows get use to the urine collection apparatus. After 7 days adaptation, liquid collection buckets were connected to plastic tubings to collect urine for 3 days. Urine specimens were taken every 6 h and preserved with enough glacial acetic acid to maintain an acid concentration of 2 or 3%. Samples were stored at -20℃ until analyzed. Fecal samples were collected via rectal grab sampling from these animals twice per day before two feedings during the 3 days sample collection period. Fecal samples from each day were pooled and stored at -20℃ for further analysis.

Milk from all cows was sampled and analyzed for melamine at day 10 after the experiment. Data indicated that there was no detectable melamine in any milk samples. From day 1 of the trial till 10 day after the trial, milk from all cows on the treatments was collected separately and disposed of.

Sample analysis

Samples were analyzed at State Laboratory of Animal Nutrition, College of Animal Science and Technology, China Agricultural University (Beijing, China) by using GC-MS. The method used here was published later by Wang et al. (2010). GC-MS analysis was performed on a 6890 GC series system coupled with a 5973 Mass Selective Detector (Agilent Technologies, Fermont, CA). CleanertTM PCX cartridges (3 cm^3), which were used for the purification of samples, were purchased from Agela Technologies (Beijing, China). The solid phase excretion system was a vacuum manifold processing station obtained from Agilent Technologies (USA). Ultrasonic bath (Kunshang, China) was used to promote sample dissolution.

Calculations and statistical analysis

Data were analyzed by one-way ANOVA followed by Duncan's multiple range tests. Significance was declared at $P < 0.05$.

RESULTS AND DISCUSSION

Effects of different levels of melamine feeding on melamine concentrations in milk, plasma, rumen fluid, urine and feces in Holstein dairy cows are presented in Table 2. Melamine was found in all samples tested. Melamine concentration in all samples generally increased when dose in the diet was increased.

Melamine concentration in milk

In agreement with Cruywagen et al. (2009)'s report, melamine appeared in milk from cows fed with melamine. Within 12 h after feeding, the dairy cows consumed diets containing melamine, and melamine concentration in milk generally increased with time within each group, with an exception of group 1, which had the highest milk melamine concentration at 6 h after melamine ingestion. Due to the milking time limitation, the real peak values of milk melamine concentrations might appear 12 h after melamine ingestion in group 2, 3 and 4. In the present study, the lowest dose (20 g/day/cow; group 1) was close to the dose (17.1 g/day/cow) used in Cruywagen et al. (2009)'s study. However, milk melamine concentration from the study group 1 (3.54 to 4.78 µg/ml) was much lower than that from Cruywagen et al. (2009)'s study. One of the possible reasons could be that pure chemical melamine was used, while Cruywagen et al. used melamine-adulterated gluten meal product. Industrial melamine applied in adulteration of feed material usually contains melamine and its analogues- cyanuric acid, ammalide and ammeline. World Health Organization (2009) has reported that increased toxicity results from combined exposure to melamine and cyanuric acid. This might explain the difference between the study results and that of Cruywagen et al. (2009).

At each sampling time, the melamine concentration in milk was generally higher as the melamine dose in the diet increased. However, the melamine concentrations measured in milk from the groups 1 and 2 had no significant difference, whereas the milk melamine concentrations of groups 3 and 4 were significantly higher (approximately two times and five times higher, respectively) than that of group 1 and 2. The disproportionate dose response of milk melamine concentration to the increasing melamine dose in the diet indicated that the animal body or mammary tissue might be able to tolerate a relative low dose of melamine, in the present study, which was lower than 40 g/day of melamine per cow.

Melamine concentration in plasma

Unlike milk melamine concentration, plasma melamine concentration at different hours from each group was affected in a similar pattern. Melamine concentration in plasma increased from 0 to 6 h, and decreased from 6 to 12 h after melamine ingestion. As melamine dose in the diet was increased, plasma melamine concentration increased at each sampling time among groups. Clearance mechanisms for melamine and its analogues have not been characterized in various animal species including dairy cattle; however, all blood concentrations of melamine (0.79 to 23.17 µg/ml) in the present study were below the safe level of 50 µg/ml proposed by the US FSIS (USDA, 2007). It seemingly indicated that the dose of melamine utilized in the present study was below the tolerable daily intake value of melamine for dairy cows. Additionally, none of our cows lost her appetite or refused her feed for the duration of the experiment.

Melamine concentration in rumen fluid

The rumen should be the main place for melamine to be dissolved. The water solubility of melamine is 3.240 g/L (20℃) and increases with temperature (Chapman et al., 1943). A mature dairy cow drinks up to 100 L of water per day, therefore, 20 to 80 g melamine could be totally dissolved in rumen. It can be noted that melamine concentration in rumen fluid increased gradually at each sampling time as melamine dose increased between groups. Melamine concentration in rumen fluid reached a peak value at 1 or 2 h in each group post melamine feeding, and decreased afterwards. Newton and Utley (1978) reported that melamine was slowly hydrolyzed in the rumen, and the mean ruminal ammonia concentration from melamine fed steers at feeding level of 45 g for over 7 days was very near to what is predicted for a low quality forage diet with no supplemental nitrogen. Based on Newton and Utley (1978)'s *in vitro* trials, ruminal microorganisms degradation and uptake for melamine may not be a significant contribution for reducing melamine from the rumen. In this study, the disappearance of melamine in rumen fluid from 1 or 2 h post feeding could be explained by melamine absorption from the rumen wall, since it is physicochemically a small polar molecule.

Melamine concentration in urine

Mast et al. (1983) investigated the metabolism, excretion and disposition of melamine by administration of a single oral dose of 0.38 mg [^{14}C] melamine to adult male Fischer 344 rats. Ninety percent of the administered dose was excreted within the first 24 h into urine and slight radioactivity appeared in the exhaled air and the feces. Only slight differences in levels of radioactivity between blood, liver or plasma were observed, radioactivity levels were much higher in the kidney and the bladder when compared to plasma (Mast et al., 1983). In agreement with their report, it was found that melamine concentration in urinary samples was greater than that from all the other kinds of samples among treatment groups, and it increased as melamine dose in the diet increased. These results indicate that melamine can be assumed to be primarily cleared by renal filtration in the lactating dairy cow.

Melamine concentration in feces

There was a significant level of melamine presented in feces from each group, and fecal melamine concentration increased as feeding dose in the diet increased. Fecal melamine level should be related to urinary and milk melamine levels. Unfortunately, milk production and total fecal excretion was not measured. Therefore, this study is unable to calculate the exact distribution of melamine in milk, urine and feces. However, according to Newton and Utley (1978)'s digestion and balance trial, 54.2% of intake melamine nitrogen appeared in urine and 40.5% of intake melamine nitrogen appeared in feces, although in different forms, including ammonia and urea. This study results also suggest that urinary and fecal excretion were the major clearance pathways for melamine in lactating dairy cows.

Conclusions

It was concluded that different doses of melamine in the diet could result in different concentrations of melamine in milk, plasma, rumen fluid, urine and feces in lactating dairy cows. Study data suggested that melamine is primarily cleared by urinary excretion, followed by fecal excretion in lactating dairy cows. Mammary tissue was apparently not a major tissue to dispose melamine, especially when fed a relatively low dose (lower than 40 g/day/cow). Notwithstanding, surveillance on raw material and animal feed production is strongly recommended, in order to avoid human melamine intake by milk consumption.

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