Hot topic: Pathway confirmed for the transmission of melamine from feed to cow's milk

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ABSTRACT

Eight lactating Holstein cows were randomly allotted to 2 groups in a trial to establish whether a pathway exists for the transmission of melamine from feed to milk. All cows received oat hay ad libitum and 15 kg of concentrate pellets per cow daily. The concentrate pellets contained either melamine-contaminated corn gluten meal of Chinese origin (melamine treatment) or locally produced melamine-free corn gluten meal (control treatment). Cows in the melamine treatment ingested 17.1 g of melamine per day. Cows were milked twice daily, and milk samples were taken once daily during the afternoon milking for melamine and milk component analyses. Melamine appeared in the milk within 8 h after first ingestion of the melamine containing pellets. Melamine concentration reached a maximum of 15.7 mg/kg within 56 h after first ingestion, with an excretion efficiency of approximately 2%. Milk solids and milk urea nitrogen were not affected by treatment. The melamine concentration dropped rapidly after changing all cows back to the control pellets, but melamine only declined to undetectable levels in the milk more than 6 d (152 h) after last ingestion of melamine. Results from the current trial are important to the feed and dairy industries because, until now, any melamine found in milk and milk products was attributed only to the deliberate external addition of melamine to these products, not to adulterated ingredients in animal feeds.

Key words: melamine, feed, milk, corn gluten meal

Melamine ($C_3H_6N_6$), or 1,3,5-triazine-2,4,6-triamine, is an industrial chemical that contains 670 g/kg of N on a molecular weight basis (Merck, 2001). Because the CP contents of feeds and foods are calculated from their N content (AOAC, 2000), melamine can artificially increase the apparent protein content when added to feed and food ingredients. Chinese authorities have recently confirmed that 6 babies had died and 296,000

had fallen ill in 2008 from drinking melamine-tainted infant formula (AllAboutFeed, 2009). Melamine, which was intentionally added to gluten meal, was also the cause of numerous pet deaths around the world in 2007 (World Health Organization, 2008a). Documented research results pertaining to melamine in production animal diets are limited. Clark (1966) reported that an intake of >10 g/d resulted in crystalluria and consequent death in sheep, whereas 7 g/d fed to sheep with an average weight of 35 kg had no adverse effects. MacKenzie (1966) also reported weight loss and mortalities when melamine was fed to sheep. Although Newton and Utley (1978) showed that melamine is not an efficient N source for ruminants, they reported that a melamine intake of 45 g/d by steers could be regarded as safe. Melamine has recently been found in some dairy cow diets in South Africa at various levels, depending on the amount of the adulterated gluten meal that was included in the dairy concentrates. No reports could be found in the literature on melamine in dairy cow diets and until now, it was generally believed highly unlikely for ingested melamine to be excreted into cow's milk.

A study was done at the Stellenbosch University (South Africa) to test the hypothesis that a pathway exists for the transmission of melamine from feed to milk; the objectives were to determine if and when melamine appeared in the milk and how long after removing melamine from the diet would milk melamine concentrations decline to undetectable levels. The trial protocol was approved by the Stellenbosch University's Animal Ethics Committee.

Cows were housed and cared for according to current ethical norms and they did not experience any discomfort at any stage of the trial. Eight lactating Holstein cows, 218 ± 9 (SE) DIM with a daily milk production of 24.6 ± 1.74 (SE) kg/d and weighing 638 ± 10.9 (SE) kg, were stratified according to milk production and then randomly allocated to 2 groups of 4 cows. Cows were housed individually in 6- × 4-m pens in a well-ventilated, semi-open barn with a cement floor. Each cow had access to a sand-bedded sleeping crate, a feeding trough, and fresh water via a ball valve-controlled water bowl.

Received January 27, 2009.

Accepted March 8, 2009.

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Cows received oat hay *ad libitum* and 15 kg/d of the experimental (**MEL**) or control (**CON**) dairy concentrate pellets, which were fed at 0630 h (8 kg/cow) and at 1630 h (7 kg/cow), immediately after the morning and afternoon milkings, respectively. On a DM basis, the concentrate consisted of ground corn (516 g/kg), soybean meal (213 g/kg), cottonseed meal (67 g/kg), corn gluten meal (69 g/kg), fish meal (25 g/kg), molasses syrup (50 g/kg), molasses meal (40 g/kg), limestone (10 g/kg), salt (5 g/kg), monocalcium phosphate (3 g/kg), and a trace mineral premix [2 g/kg; Advit Animal Nutrition SA (Pty) Ltd., Kempton Park, South Africa]. The concentrate had a calculated CP content of 250 g/kg of DM and an NDF content of 190 g/kg of DM. The MEL and CON pellets were therefore identical in physical composition, except that the CON pellets contained 69 g/kg of a locally produced, melamine-free corn gluten meal, whereas the MEL pellets contained the same amount of a Chinese corn gluten meal with 15,117 mg/kg of melamine (by liquid chromatography tandem MS analysis, determined in our lab). Because of the sensitivity of this issue and a confidentiality agreement, the supplier of the contaminated corn gluten meal cannot be disclosed. Total mixed rations and dairy concentrates in South Africa would, respectively, not contain more than 50 g/kg and 70 g/kg of corn gluten meal. In an attempt to ensure that we had the best chance of detecting significant levels of melamine in the milk, it was decided to include the maximum amount of corn gluten meal in the concentrate pellets which, in the end, came to 69 g/kg. The adulterated corn gluten meal had a CP content of 674 g/kg (DM basis), and previous microscopic analysis (C. W. Cruywagen; unpublished data) revealed that it consisted of wheat starch, wheat bran, corn bran, corn gluten feed, corn gluten meal, urea, melamine, and colorants. One tonne of each concentrate diet was manufactured, pelleted, and bagged in 50-kg bags. Analyses of samples taken randomly from 7 bags of each treatment indicated that the CON pellets had a CP content of 251 g/kg, and that of the MEL pellets was 263 g/kg. The difference was due to the higher CP content of the adulterated Chinese corn gluten meal. The analysis also confirmed that the CON pellets contained no detectable melamine (i.e., <0.001 mg/kg), whereas the MEL pellets contained 1,142 mg/kg of melamine, which compared well with the calculated value of 1,043 mg/kg. Ingestion of 15 kg of the MEL pellets would thus result in a melamine intake of 17.1 g/d per cow. Calculations based on the work of Clark (1966) and Newton and Utley (1978) would suggest that intake of 0.13 to 0.16 g/kg of live weight should not have a detrimental effect on the health of ruminant animals. On this basis, a 650-kg lactating dairy cow should be able to ingest 85 to 100 g/d of melamine

without apparent ill effects. In the current trial (intake of 17 g/d of melamine per cow), we therefore assumed that cow health would not be compromised.

Before commencement of the trial, milk of all the cows was tested and no melamine was detected in any of the samples. At the onset of the trial, all cows received the melamine-free CON pellets for 2 d to ensure that they ate the feed and that the milk tested free of melamine. From the third day, the 4 cows in the experimental group received the MEL dairy pellets. Cows continued to receive the respective treatment diets for 8 d to determine the asymptote milk melamine level. The MEL group was then changed back to the control diet for another 5 d. Refusals were weighed back daily before the a.m. feeding, but refusals consisted of oat hay alone, as all the cows ate all their concentrate pellets each day. Cows were milked twice daily at 0500 and 1500 h, and milk yield was recorded individually at each milking throughout the trial. Because of the number of samples and the high cost of analysis, milk was sampled only once a day, during the p.m. milking. Milk samples were split into subsamples to be stored at -18°C or preserved with potassium dichromate until analyzed. The frozen samples were analyzed for melamine, and the dichromate-preserved samples were tested for milk protein, fat, lactose, and MUN content with the aid of a MilkoScan FT 6000 (Foss, Hillerød, Denmark).

For the duration of the trial, milk from cows on the MEL diet was collected separately and discarded by washing it down the drain. Any milk that could possibly be contaminated with melamine was therefore never mixed with the other milk.

On the last day of the MEL treatment, spot urine samples were taken from all the cows. The urine samples were stored at -18°C until analyzed for melamine. After the trial had ended and milk from all the cows contained no detectable levels of melamine, the cows were reinstated into the Stellenbosch University's dairy herd.

For melamine analysis, an adapted method of Shai et al. (2008) was used. Feed samples were prepared by grinding through a 1-mm screen, and 1-g quantities were extracted with 10 mL of acetonitrile (50%). Milk and urine samples were diluted 1:1 in 0.2 M perchloric acid and centrifuged at $4,500 \times g$ for 5 min. Cation-exchange solid-phase extraction cartridges (Phenomenex Strata SCX; 55 μm , 70 Å, 500 mg/3 mL, supplied by Separations, Randburg, South Africa) were conditioned with 6 mL of methanol, followed by 6 mL of water. The supernatants of the milk samples (0.5 mL) were loaded onto the cartridges together with 100 μL of a 0.5 mg/L stable isotope-labeled melamine ($^{13}\text{C}_3\text{H}_6^{15}\text{N}_3$) internal standard solution (Cambridge Isotope Laboratories Inc., Andover, MA). Thus, 0.05 μg of labeled melamine

Figure 1. Milk production and melamine concentration of milk from cows that ingested 15 kg/d of a pelleted dairy concentrate that contained 1,142 mg/kg of melamine. First ingestion of melamine was 8 h before the afternoon milking on d 3 of the trial. Daily melamine intake was 17.1 g/cow.

was loaded onto each cartridge. The cartridges were washed with 0.1 *N* HCl (6 mL) followed by methanol (6 mL) and aspirated under vacuum for 1 min; the melamine was then eluted with 6 mL of ammonium hydroxide:methanol:dichloromethane (1:5:5) into a clean tube. The extracts were dried under a stream of nitrogen and resuspended in 1 mL of acetonitrile (50%). Samples were analyzed by liquid chromatography tandem MS on a Waters API Quattro Micro triple quadrupole mass spectrometer coupled to a Waters 2690 HPLC (Waters Corp., Milford, MA). The limit of detection of the method is 0.001 mg/kg for feed samples and 0.005 mg/kg for milk and urine samples. The average recovery for fortified milk was 102% spiked at levels of 0.5 mg/kg and 5 mg/kg. The internal standard was used to correct for dissimilarities in recovery rates at different concentrations.

Milk composition data were subjected to a one-way ANOVA using the GLM procedure of SAS (SAS Institute, 1999). Significance was declared at $P < 0.05$. Because only the MEL treatment resulted in milk melamine, no statistical analyses were done on melamine data, except for standard error bars that were included in Figure 1 to indicate variation in daily milk production and melamine content over time.

Results (Figure 1) confirm the existence of a pathway of melamine from feed to milk. Milk from the CON

treatment never contained detectable melamine, but melamine appeared in milk from cows in the MEL treatment 8 h after first ingestion of the melamine-containing diet. Melamine concentration increased rapidly and reached a maximum within 56 h after first ingestion of the experimental diet. The exact time of first appearance of melamine and the time to reach maximum concentration in milk are still unknown, because in our trial, milk was sampled at 24-h intervals during the afternoon milkings, which were 8 h after the morning feeding. In this trial, the melamine excretion efficiency (g of melamine ingested/g of melamine excreted into milk) was 0.5% at 8 h after first ingestion. When the maximum concentration was reached, the excretion efficiency increased to 2.1%. After the maximum melamine concentration had been reached, values fluctuated somewhat over the next 5 d while cows were on the MEL treatment. This fluctuation was probably related to daily variations in milk production (Figure 1). Afternoon milk yields showed the same fluctuation patterns as total milk and it was thus accepted that calculations based on the analysis of afternoon milk only were probably representative of the pattern of total melamine concentrations in milk. The mean melamine excretion efficiency during this period ranged from 1.7 to 2.1%. When cows were switched back to the CON pellets, the concentration of milk melamine

Table 1. Milk production and composition of cows in the melamine trial

Item	Treatment ¹		SEM	<i>P</i> -value
	MEL	CON		
Daily milk yield (kg)	24.9	24.3	1.22	0.770
Milk fat (g/kg)	32.6	33.8	4.12	0.845
Protein (g/kg)	34.7	36.3	2.60	0.675
Lactose (g/kg)	47.6	47.1	1.42	0.824
Total solids (g/kg)	118.7	124.4	5.92	0.518
MUN (mg/100 mL)	16.8	17.3	0.62	0.583

¹MEL = melamine treatment: cows received oat hay ad libitum and 15 kg/d of a concentrate pellet containing 1,142 mg/kg melamine; CON = control diet: cows received oat hay ad libitum and 15 kg/d of a melamine-free concentrate pellet.

responded rapidly and dropped 39% within 8 h and 85% within 32 h. After that, milk melamine levels declined and melamine could not be detected in milk 152 h after changing back to CON pellets. It is speculated that dietary melamine concentration would have an effect on melamine excretion efficiency as well as on the time for melamine to disappear from the milk once a contaminated diet had been removed.

The implications of our results are important to the dairy industry. The US Food and Drug Administration recently announced that levels of melamine <2.5 mg/kg in foods other than infant formula do not raise public health concerns (CNN, 2008). They set the maximum level for infant formula at 1 mg/kg. If a dairy cow concentrate contained 40 mg/kg of melamine and it was fed at a level of 12 kg/d to cows with a similar milk production as those in the current study (25 kg/d), then our results would suggest an expected milk melamine concentration of 0.4 mg/kg. This might be seen as a “safe” level, but when such milk is converted to milk powder, the melamine level would be concentrated approximately 8-fold to 3.2 mg/kg, which is above the accepted level, especially when the resultant powder is to be used in infant formula.

Milk composition was not affected by treatment (Table 1). No literature could be found on the ruminal degradability of melamine, but it is expected that melamine would be degraded to some extent. The question was therefore asked whether dietary melamine would affect MUN. It is clear from Table 1 that MUN content was not affected by melamine ingestion.

Excretion via milk accounted for only 2% of the ingested melamine. The absorption rate of melamine from the digestive tract is still unknown, but documented evidence of kidney damage and melamine crystalluria (Clark, 1966) would suggest that the major excretion pathway is via urine. According to the report of an expert meeting of the World Health Organization (2008b), melamine and cyanuric acid are rapidly

absorbed in monogastric animals and excreted unmetabolized in the urine. It is postulated that the same happens in ruminants, as we could find no documented evidence of significant melamine degradation in the rumen. Melamine can also react with cyanuric acid on a 1:1 basis to form spoke-like melamine cyanurate crystals from aqueous solutions. These crystals have been implicated as a causative toxicity agent in the Chinese protein export contamination and the 2007 pet food recall (He et al., 2008). In the current trial, analyses of urine samples taken from all the cows on the last day of the MEL treatment indicated that urine of cows in the MEL treatment contained 452.3 ± 21.3 (SE) mg/kg of melamine, whereas no melamine was detected in the urine of cows in the CON treatment. The concentration of melamine in the urine of cows fed MEL supports the hypothesis that urine is the major route of melamine excretion in lactating dairy cows.

We concluded that a pathway exists for the transmission of melamine from feed to milk and the kinetics suggest that milk melamine concentration responds rapidly to the introduction of melamine-contaminated feed. However, despite a rapid initial decline in melamine concentration upon removing melamine from the feed, it can take 6 d or longer before the milk is completely free of melamine again. This is probably related to the passage rate of melamine-containing feed from the rumen. Dietary melamine concentration and feeding behavior of cows would likely affect milk melamine concentration and excretion efficiency. Apparent complete clearance of melamine from milk would depend on the sensitivity of analytical methods and would probably also be affected by dietary melamine concentration. Results from the current trial have implications for the dairy industry because, until now, any melamine found in milk and milk products was attributed only to the deliberate external addition of melamine to these products and not to adulterated ingredients in animal feeds.

ACKNOWLEDGMENTS

The authors express gratitude to Tanqua Feeds (Riviersonderend, South Africa) for sponsoring the feed and mixing the concentrate diets according to our specifications, and Ivan Stevens (farm manager), Sam Pietersen, and James Williams, all at the Welgevallen Experimental Farm of the Stellenbosch University, for caring for the animals. The Hennie Steenberg Trust Fund is acknowledged for financial assistance.

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