A Survey on Occurrence of Melamine and Its Analogues in Tainted Infant Formula in China

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Objective To investigate the occurrence and concentrations of melamine and its analogues in tainted infant formula and to identify the etiologic factors for the urinary stones epidemic in infants and young children in China in 2008. **Methods** Sanlu infant formula samples were collected from families of the affected children in Gansu province, and markets in Gansu and Hebei provinces and Beijing city. Melamine and its analogues, including cyanuric acid, ammeline, and ammelide were measured by gas chromatography/ tandem mass spectrometry. **Results** High prevalence and concentrations of melamine were found in Sanlu infant formula samples, with low concentrations of cyanuric acid, ammeline and ammelide. Melamine were detected in 87 out of 111 Sanlu infant formula samples with a range of 118 to 4 700 mg/kg, **Conclusion** The results provide strong evidence for melamine as the etiological factor for the urinary stones epidemic in infants and young children in China in 2008.

Key words: Melamine; Cyanuric acid; Dairy products; Powdered infant formula; China

INTRODUCTION

Melamine with a chemical formula of $C_3H_6N_6$ is an organic compound that consists of 66% nitrogen. It is produced in a large amount primarily for use in the synthesis of melamine formaldehyde resins for the manufacture of laminates, plastics, coatings, commercial filters, glues or adhesives, and dishware, and kitchenware[1]. Melamine was used as a source of nonprotein nitrogen (or NPN) in cattle feed from 1958 to 1978 in China[2]. While melamine alone is of low toxicity, it could form insoluble crystals in combination with cyanuric acid, leading to the formation of kidney stones[3-7], which may cause kidney failure and ultimately death, particularly in vulnerable individuals such infants and young children. For adults, melamine is only dangerous in very high concentrations. In this regard, the US Food and Drug Administration (FDA) has set a tolerable daily intake (TDI) of 0.5 mg/ kg body weight[4].

Since the spring of 2008, an increasing number of kidney stones in infants were noticed by pediatricians in Gansu, Hebei, Beijing, and other cities/provinces in China[1,8]. This was followed by a survey to identify the cause of this unusual epidemic. According to the information released by the Ministry of Health, the People's Republic of China (MOH), 294 000 infants and young children in China had been diagnosed to have urinary tract stone by the end of November 2008. Although most patients had no symptoms and signs, acute renal failure occurred in a small proportion of patients. More than 50 000 infants had been hospitalized, and six deaths had been confirmed[9].

Investigation revealed that the infant formula made from adulterated raw milk was the cause of the urinary tract stone epidemic. During the adulteration, melamine, so-called "protein essence", was illegally added into raw milk to falsely increase the protein content of milk after dilution with water[1,8]. In September 2008, it was confirmed that the infant formula products from Sanlu Company, one of the largest milk powder manufacturers in China, contained high levels of melamine[1,8]. This finding is

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consistent with a large number of cat deaths that had occurred in USA in 2007 due to kidney failure caused by consumption of melamine-contaminated pet foods imported from China[4-7]. This paper reports the results of the survey on the occurrence of melamine in Sanlu infant formula during the melamine crisis in 2008.

METHODS

Sampling

A total of 111 Sanlu infant formula samples were collected from markets in Beijing and Gansu province, including some samples from families with the affected infants in Gansu province. In addition, 38 other infant formula samples (non-Sanlu) were collected from markets in Gansu province to compare with the 52 Sanlu samples also collected from Gansu province. Furthermore, 87 raw materials used for raw milk adulteration were collected from Hebei province where Sanlu Company was located.

Method of Analysis

Melamine and its analogues were quantified using the method as we previously described[10]. Briefly, melamine and its analogues including cyanuric acid, ammeline and ammelide were extracted from the samples using a solvent mixture of diethylamine, water and acetonitrile (10/40/50, v/v/v,). After the extracts were evaporated to dryness by a gentle nitrogen stream, the target compounds were derivatized by the silylating reagent. Finally, melamine and its analogues were determined by gas chromatography/ tandem mass spectrometry with 2, 6-diamino-4-chloropyrimidine (DACP) being used as an internal standard. The reported limit of quantification (LOQ) was 0.05 mg/kg and the reported limit of detection (LOD) was 0.01 mg/kg.

RESULTS

Melamine in Sanlu Infant Formula Samples

The occurrence of melamine in Sanlu infant formula collected from markets in Beijing and Gansu province, including samples from families with the affected infants in Gansu province, is listed in Table 1. Melamine was detected in 87 out of 111 samples with varying concentrations. No melamine was detected in the other 24 samples using the reported limit of quantification (LOQ) of 0.05 mg/kg as a cut-off number. Of the 87 melamine positive samples, 51 samples had a melamine concentration higher than 1 000 mg/kg (the median). The lowest and the highest concentrations were found to be 118 and 4 700 mg/kg, respectively.

TABLE1

Detection of Melamine in Sanlu Infant Formula Samples (*n*=111)

Melamine Conc. (mg/kg)	No. of Samples	Mean (mg/kg)	Median (mg/kg)	70th Percentile (mg/kg)	90th Percentile(mg/kg)
<0.05	24	-	-	-	-
0.05-1000	32	506	555	652	900
1000-1900	22	1439	1500	1700	1840
1900-2600	22	2212	2200	2300	2560
>2600	11	3453	3000	4000	4500

Notes. The 50th, 70th, and 90th percentiles of the 111 samples are 1 000, 1 900, and 2 600 mg/kg, respectively. The reported LOQ is 0.05 mg/kg.

Comparison of Melamine Levels between Sanlu and Non-Sanlu Infant Formula Samples Collected from Gansu Province

Higher concentration of melamine was detected in the Sanlu infant formula samples compared with that in the other samples. In general, most of the infant formula samples from other producer had very low concentration of melamine. The mean, median, 90th percentile, 25th percentile and 10th percentile of melamine in Sanlu and non-Sanlu infant formulas collected from Gansu Province are shown in Table 2.

TABLE 2

Melamine Content in Sanlu and Non-Sanlu Infant Formula Samples

	No. of Samples	Mean (mg/kg)	Median (mg/kg)	90th Percentile (mg/kg)	25th Percentile (mg/kg)	10th Percentile (mg/kg)
Sanlu	52	1673.58	1700.00	2880.00	980.00	24.00
Non-Sanlu	38	4.06	0.01	8.80	0.01	0.01

Note. The undetectable level was set as 1/2 reported LOD 0.01 mg/kg.

Melamine and Its Analogues in Sanlu Infant Formula Samples Collected from Gansu Province

The mean, median and 90th percentile of melamine as well as its analogues in Sanlu infant formulas collected from Gansu Province are shown in Table 3. On an average, the ratio of melamine to its analogues was 1 045 for melamine to cyanuric acid, 984 for melamine to ammeline and 577 for melamine

to ammelide.

TABLE 3

Concentrations of Melamine and its Analogs in Sanlu Infant Formula Samples Collected from Gansu Province (n=52)

	Mean (mg/kg)	Median (mg/kg)	90th percentile (mg/kg)
Melamine	1 673.6	1 700.0	2 880.0
Cyanuric Acid	1.6	1.1	3.4
Ammeline	1.7	1.3	4.3
Ammelide	2.9	2.1	8.1

Note. The undetectable level was set as 1/2 reported LOD 0.01 mg/kg.

Determination of Melamine and Its Analogues in Raw Materials Used for Adulteration

The 56 samples of raw materials (not labeled) used for adulteration at the milk collection stations were also analyzed for melamine and its analogues. Among them, 31 samples were found to have high concentrations of melamine. In these samples, 15 samples had a mean of 178 571 mg melamine/kg (Table 4). In addition, the ratio of melamine to ammeline or ammelide was similar to that in Sanlu infant formula samples. However, the ratio of melamine to cyanuric acid in raw material was significantly lower than that in Sanlu infant formula samples.

TABLE 4

Results of Melamine and Its Analogues in Raw Materials Used for Adulteration (n=15)

	Mean (mg/kg)	Median (mg/kg)	90th Percentile (mg/kg)
Melamine	178 570.7	188 000.0	339 000.0
Cyanuric Acid	7.0	3.2	14.7
Ammeline	116.7	14.9	289.9
Ammelide	354.6	292.8	452.8

Note. The concentration for the samples with undetectable analyte was set as 1/2 LOD.

DISCUSSION

Melamine is a chemical used in the manufacture of amino resins and plastics. Neither approval for use of melamine into food is granted by any countries, nor recommendation is given in the Codex Alimentarius. The illegal addition of melamine into milk and feed is to increase falsely the apparent protein content of food products because the commonly used methods for protein measurement cannot distinguish between nitrogen from protein and non-protein sources. Addition of melamine would result in incorrectly high protein measurements and provide an economic incentive for its illegal use. Melamine has been also found in feeds of animals including chickens, hogs, and fish[6-7]. In China, where adulteration had occurred, water had been added into raw milk to increase the volume. As a result of this dilution, the milk had a lower protein concentration and the companies using the milk for further production (e.g. of powdered infant formula) would produce the products which had a low protein content but high melamine.

Humans could be also exposed to melamine and its analogues from a number of other sources. Firstly, one of the sources would be from breakdown of the pesticide cyromazine, which is approved for use in many countries. Secondly, migration from melamine resin containers to food has been reported. Thirdly, melamine could be introduced into human diets as it may carry over from the illegal use of melamine in animal feed or feed ingredients although no quantitative data exist. In this regard, some data have shown that the carry-over occurs from feed to products of animal origin including milk, eggs, meat, and fish. Lastly, contamination of melamine into the food chain as a baseline level is possible as it is present in the environment and the widespread use of melamine-containing materials[1,12].

China has issued an interim control limit in powdered infant formula (1 mg/kg) and in other foods (2.5 mg/kg). The US Food and Drug Administration (FDA) sets 2.5 mg per kg as the concern level, while Hong Kong, China and New Zealand also set a safe limit in food products of 2.5 mg per kg, though Hong Kong has lowered the level for children under 3 and pregnant or lactating women to 1 mg per kg. Compared with 1 mg per kg, the melamine contents of adulteration samples in Table 1 exceeded the limit level notably. The baseline level refers to the amount of melamine from the unintentional use, while the adulteration level is that of melamine in food that result from the intentional addition of melamine or the unapproved use or misuse of melamine and any substances that can degrade to form melamine[1,12]. The present results showed the Sanlu infant formula samples had an unacceptable level of melamine. Furthermore, the high level of melamine was found in the raw milk and raw adulterated materials obtained from the milk collection station. The dietary exposure on the basis of the consumption of melamine adulterated infant formula in China at the median levels of melamine reported in the most contaminated brand was estimated to range from 8.6 to 23.4 mg/kg body weight per day. This is about 40-120 times of the TDI of 0.2 mg/kg body weight set by WHO[1,12].

The present results are in agreement with those on the infant formula samples collected prior to September 14, 2008 by the General Administration of Quality Supervision, Inspection and Quarantine of China (AQSIQ), demonstrating that the melamine levels ranged between 118 and 4 700 mg/kg. During the melamine-tainted infant formula event, the AQSIQ inspected all types of milk and milk products in the market, including infant formula, liquid milk and other milk powders. AQSIQ had reported that the levels of melamine in the above dairy products (including infant formula) ranged between 0.09 to 6 196.61 mg/kg[11]. The reported LOQ was 0.05 mg/kg, and those samples with a value higher than the LOQ was considered positive. For infant formula, 69 sample lots from 22 producers were positive with the maximum of 2 563 mg/kg in 491 lots from 109 producers prior to September 14, 2008. After then, all the 1 645 lots from 78 producers were found to be negative. For the other milk powders, 31 lots from 20 producers were positive with a maximum of 6 196 mg in 265 lots from 154 producers prior to September 14, 2008, thereafter, all 2 310 lots from 184 enterprises were negative. For the liquid milk, 24 lots from 3 producers were positive with a maximum of 8.6 mg/kg in 1 202 lots from 5 producers prior to September 14, 2008, and all 2 310 lots from 184 producers thereafter were negative.

Commercially produced melamine may contain its analogues, such as cyanuric acid, ammelide and ammeline. Melamine can form some self-associating and high molecular weight complexes through intramolecular networks of hydrogen bonds and π-π aromatic ring stacking interactions with cyanuric acid and other analogues as well as uric acid and other cyclic imide-containing biomolecules. Animal data had shown that melamine alone could lead to formation of kidney stones after a high dose exposure. Evidence from an earlier outbreak of acute renal failure in cats and dogs associated with contaminated pet food suggested that a combination of melamine and cyanuric acid could cause renal toxicity. Subsequent experimental studies had shown that when animals were fed a mixture of melamine and cyanuric acid, crystals were formed in the tubules of the kidneys causing renal damage and renal failure. However, in the current melamine crisis in China, the presence of cyanuric acid had not yet been confirmed. Chinese infant formula reportedly contained levels of cyanuric acid, ammeline and ammelide that were only about 0.1% of melamine. The lower levels of these analogues were found to be present in contaminated wheat gluten and rice protein concentrate ingredients used in the production of pet foods during the 2007 melamine contamination incident in the United States, Canada and South Africa. The melamine added into adulterated milk for at least some of the infant formula produced in China that caused renal illnesses during the 2008 incident appeared to be relatively pure. This is in consistent with the findings that the major composition of the urinary tract stones from the affected patients were melamine and uric acid[13], which is very different from the melamine – cyanuric acid stones from the cat events[1,12].

PRELIMINARY CONCLUSION

In conclusion, high prevalence and concentrations of melamine were found in Sanlu infant formula samples, suggesting that melamine tainted infant formula was the cause of urinary tract stone epidemic in China. It was found that Sanlu infant formula contained very low concentrations of cyanuric acid, ammeline, and ammelide with a profile similar to that in raw materials used for milk adulteration. It deems necessary to develop a simple, specific, rapid and cost-effective method for protein quantification in prevention of melamine adulteration in the future.

REFERENCES

1. World Health Organization (WHO). Expert meeting to review toxicological aspects of melamine and cyanuric acid, Ottawa, Cnanda, 1-4 December 8, 2008, Executive Summary. (Accesed at http://www.who.int/foodsafety/fs_management/Exec_Summary_melamine.pdf)
2. Liu Q S, Wang J Q (2008). Melamine toxicity in animals. *China Animal Husbandry & Veterinary Medicine* **35**(10), 5-7 (In Chinese).
3. Brown C A, Jeong K S, Poppenga R H, *et al*. (2007). Outbreaks of renal failure associated with melamine and cyanuric acid in dogs and cats in 2004 and 2007. *J Vet Diagn InVest* **19**, 525-531.
4. U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition. Pet food recall (melamine)/tainted animal feed. July 23, 2007. http://www.fda.gov/oc/opacom/hottopics/petfood. html (accessed 1/11/08).
5. McPheron T. Melamine and cyanuric acid interaction may play part in illness and death from recalled pet food. May 1, 2007. http://www.avma.org/press/releases/070501_petfoodrecall.asp (ac- cessed 1/14/08).
6. U.S. Food and Drug Administration. FDA asks feed manufacturers to avoid ingredients containing melamine. May 30, 2007. http://www.fda.gov/bbs/topics/NEWS/2007/NEW01643.html (accessed 1/11/08).
7. Burns K (2007). Hogs chickens ate pet food containing adulterants. *J Am Vet Med Assoc* **230**, 1603.
8. Chen J S (2009). A worldwide food safety concern in 2008-melamine-contaminated infant formula in China caused urinary tract stone in 290000 children in China. *Chinese Medical Journal* **122**(3), 243-244.
9. Ministry of Health P. R. China. The special topic of infant formula powder case on Sanlu (24 Jan 2009). accesed at http://www.moh.gov.cn/publicfiles/business/htmlfiles/wsb/pmtxwbd/200901/38923.htm
10. Miao H, Fan S, Zhang L, *et al*. (2009). Simutaneous determination of melamine, ammelide, ammeline, and cyanuric

acid in milk and milk Products by gas chromatography – tandem mass spectrometry. *Biomedical and Environmental Sciences* **22**(2), 87-94.
11. World Health Organization (WHO). Melamine contamination. (accesed at http://www.who.int/foodsafety/fs_management/infosan_events/en/index1.html).
12. World Health Organization (WHO). Expert meeting to review toxicological aspects of melamine and cyanuric acid, Ottawa, Canada, 1-4 December 8, 2008, Overall conclusion and recommands. (Accesed at http://www.who.int/foodsafety/fs_management/conclusions_recommendation.pdf)
13. Sun N, Shen Y, Sun Q, *et al* (2008). Melamine related urinary calculus and acute renal failure in infants. *Chin J Pediatr* **46**(11), 810-815. (In Chinese)

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