Effects of Serum Proteins on *In Vitro* Melamine-Cyanurate Crystal Formation

Sarawut TAKSINOROS¹⁾ and Hideo MURATA^{2)*}

¹⁾Division of Engineering (Biotechnology), Graduate School of Engineering, Kyoto Sangyo University, Kamigamo, Kita-ku, Kyoto, Kyoto 603-8555, Japan

²⁾Faculty of Life Sciences, Kyoto Sangyo University, Kamigamo, Kita-ku, Kyoto, Kyoto 603-8555, Japan

(Received 26 March 2012/Accepted 4 July 2012/Published online in J-STAGE 18 July 2012)

ABSTRACT. Melamine toxicity is recognized as a distinct form of renal failure due to occlusion of the renal tubules by the compound melamine and its deaminated derivative, cyanuric acid. The morphology of melamine-cyanurate crystals *in vivo* differs from that *in vitro*, being rounded in the former case but needle-like in the latter. The reasons for this difference in morphology between *in vivo* and *in vitro* crystals remain unknown. In the present study, we investigated the *in vitro* effects of several possible intra-renal factors, i.e., pH and serum and urinary proteins, on the morphology of melamine-cyanurate crystals in order to clarify what might be responsible for the formation of rounded urolites *in vivo*. We found that serum proteins, such as fetal bovine serum, bovine serum albumin and bovine gamma-globulin, can alter the morphology of melamine cyanurate, turning it into rounded crystals. The urinary protein beta-2-microglobulin had a less pronounced effect. The crystal morphology was unaffected by pH. Based on the present *in vitro* findings and known clinical data, we suggest a putative protein-related model for melamine-cyanurate formation in the kidney.

KEY WORDS: formation, melamine cyanurate, morphology, round-type crystal, serum proteins.

doi: 10.1292/jvms.12-0138; *J. Vet. Med. Sci.* 74(12): 1569–1573, 2012

Recently, melamine toxicity has been widely reported in humans and other species, having an adverse effect on the renal system [1, 4, 9, 17, 25]. Human infants who consume melamine-contaminated powder formula develop renal dysfunction and renal failure due to melamine-triggered occlusion of the renal tubules [18, 23].

Melamine is a nitrogen-rich heterocyclic triazine normally used primarily in the synthesis of melamine-formaldehyde resins, but sometimes it is added to dairy products to boost the apparent protein level, and subsequent consumption of the adulterated products can be harmful [3, 7, 18, 19]. Although melamine alone has low acute toxicity, simultaneous exposure to melamine and its deaminated derivative cyanuric acid has an affect that is more toxic than exposure to each compound individually [13, 14, 18]. The compound formed by melamine and cyanuric acid, melamine cyanurate, accumulates in the proximal and distal renal tubules [5], where it causes obstruction and thus leads to kidney malfunction [15].

In vitro, melamine and cyanuric acid individually form clear solutions in water, but when mixed they form an opaque suspension of melamine cyanurate complex consisting of needle-like crystals that can be discerned by light microscopy [22]. *In vivo*, however, the crystals are reported to have a different shape, being rounded and brown, and found in the kidney or urine in some species [9]. The reasons for this difference in morphology between *in vitro* and *in vivo*

conditions remain unclear.

In the present study, we investigated the *in vitro* effects of several possible intra-renal factors, i.e., pH and serum and urinary proteins, on the morphology of melamine-cyanurate crystals in order to clarify what might be responsible for the formation of rounded urolites *in vivo*.

MATERIALS AND METHODS

Preparation of melamine and cyanuric acid solutions at various pH values: Solutions of melamine and cyanuric acid at 10 mmol/l were prepared at three pH values (pH 5.6, 7.0 and 8.1) using 100 mmol/l sodium phosphate- buffer (Na₂HPO₄-NaHPO₄, Wako Pure Chemical Industries, Ltd., Osaka, Japan). A compact pH meter (twin pH meter B-212, Horiba, Ltd., Kyoto, Japan) was used for confirmation and monitoring of the pH.

Preparation of serum and urinary proteins in the melamine and cyanuric acid solutions: The serum and urinary proteins used were fetal bovine serum (FBS, GIBCO, Invitrogen, Grand Island, NY, U.S.A.), bovine serum albumin (BSA, MW=66 kDa, Wako Pure Chemical Industries, Ltd., Osaka, Japan), bovine gamma-globulin (BGG, MW=ca.150 kDa, MP Biomedicals, LLC, Illkirck, France) and beta2-microglobulin (B2M, MW=12 kDa, Sigma, St.Louis, MO, U.S.A.). They were added at concentrations of 0.1, 0.5, 1, 5 and 10% (w/v) to each of the 10 mmol/l melamine or cyanuric acid solutions in Hanks' buffered salt solution [HBSS (–), Nacalai Tesque Inc., Kyoto, Japan]. The maximum level (10% w/v) was set based on the concentration of plasma proteins (6–8%) in several animal species [2].

Melamine cyanurate (M-C) crystal formation: The M-C crystals were formed by mixing the melamine and cyanuric

*CORRESPONDENCE TO: MURATA, H., Faculty of Life Sciences, Kyoto Sangyo University, Kamigamo, Kita-ku, Kyoto, Kyoto 603-8555, Japan.

e-mail: hmurata@cc.kyoto-su.ac.jp

©2012 The Japanese Society of Veterinary Science

acid solutions (ratio 1:1 on a molar basis), and the physical appearances (opacity, color and pH) of the suspensions were observed using a light microscope (Nikon E200, Tokyo, Japan).

Ultra-filtration procedure: Ultrafiltration with a 10 kDa cut-off, simulating the selective glomerular filtration that occurs *in vivo*, was used to separate the serum proteins (FBS, BSA and BGG) from the solution of melamine or cyanuric acid. The melamine or cyanuric acid solution, containing each of the serum proteins at a concentration of 5%, was centrifuged ($14,000 \times g$ at 20°C for 10 min) in an ultrafiltration unit (Amicon Ultra-0.5 ml 10K, Millipore Ireland Ltd., Cork, Ireland). The morphologies of the filtrate mixtures [ratio 1:1 (v/v)] of melamine and cyanuric acid solutions were evaluated microscopically in comparison with those of the mixtures of non-filtered solutions.

RESULTS

Effect of pH on M-C crystal morphology: All of the M-C mixtures at various pH values were opaque, and the crystals formed showed the same needle-like morphology. No rounded crystals were confirmed (Table 1).

Effects of serum and urinary proteins on M-C morphology: The M-C crystals changed to a rounded shape when the solutions contained serum proteins. The crystal morphology and the fluid color, used as a rough pH indicator, differed slightly among the protein groups (Table 2). The crystal morphology of BSA-M-C and FBS-M-C was elongated at a concentration of 1%, but BGG-M-C showed a round sur-

Table 1. Effect of pH on M-C crystal morphology

pH	Physical appearances	
	Crystal morphology	Opacity
5.6	Needle-like	Opaque
7.0	Needle-like	Opaque
8.1	Needle-like	Opaque

Fig. 1. M-C crystal morphology at different concentrations of bovine gamma-globulin (BGG).

face and elongated shape at concentration of 0.5 and 0.1%, respectively (Fig. 1). The M-C crystal morphology became rounded in the presence of high serum protein concentration, but changed to a larger and longer form in the presence of a low serum protein concentration, while B2M-M-C crystals did not adopt a rounded shape at any B2M concentration (Fig. 2). The changes in fluid color and pH appeared to depend on the serum protein concentration (Table 2).

Effects of ultra-filtration of serum proteins on M-C crystal morphology: With regard to ultra-filtration treatments,

Table 2. Effects of serum and urinary protein concentration on M-C crystal morphology

Proteins	Physical appearances				
	Concentration (w/v %)	M-C crystal morphology	Liquid		
			Opacity	Color	pH
FBS (serum)	0.1	Round-Needle-like	√	Dark-Pink	9.5
	0.5	Long-round	√	Dark-Pink	9.3
	1	Short-elongated	√	Dark-Pink	9.3
	5	Rectangle-round	√	Dark-Pink	9.2
	10	Rectangle-round	√	Dark-Pink	8.8
BSA (serum)	0.1	Round-Needle-like	√	Dark-Pink	9.3
	0.5	Long-round	√	Dark-Pink	8.8
	1	Short-elongated	√	Pink	8.2
	5	Square-round	√	Dark-Yellow	7.3
	10	Square-round	√	Dark-Yellow	7.1
BGG (serum)	0.1	Short-elongated	√	Pink	8.8
	0.5	Round	√	Pink	8.6
	1	Sphere-round	√	Pink	8.6
	5	Sphere-round	√	Pink	7.6
	10	Sphere-round	√	Pink	7.2
B2M (urine)	0.1	Needle-like	√	Pink	—
	0.5	Round-Needle-like	√	Pink	—
	1	Thin-Long-round	√	Pink	—
	5	Thin-elongated	√	Pink	—
	10	Short-elongated	√	Pink	—

√: Opaque, —: Not determined due to the small volume of the mixture.

Fig. 2. Effects of serum proteins and their concentrations on M-C crystal morphology (an illustration). Size (length $\times$ width): 10 randomly selected crystals were used for calculation.

Table 3. Effect of ultra-filtration on M-C crystal morphology

Serum protein	M-C crystal morphology	
	Control	Filtrate
FBS	Rectangle-round	Needle-like
BSA	Square-round	Needle-like
BGG	Sphere-round	Needle-like

similar results were obtained when 5% BSA, 5% FBS and 5% BGG mixtures were used. The M-C crystal morphology obtained from the control solution (without ultra-filtration) was rounded, but that obtained from all of the filtered mixtures was needle-like (Table 3). In addition, rounded M-C crystals were formed when serum protein was mixed before combination with melamine and cyanuric acid. The serum proteins failed to change the needle-like M-C crystals to the rounded form if they were added after the melamine and cyanuric acid had been combined under protein-free conditions (Table 4).

Table 4. Effect of the order of combination on M-C crystal morphology

Combination order			M-C crystal morphology
1st	2nd	3rd	
M	C	—	Needle-like
M	C	BGG	Needle-like
M	BGG	—	Not found
M	BGG	C	Sphere-round
C	BGG	—	Not found
C	BGG	M	Sphere-round

M: Melamine (10 mmol/l), C: Cyanuric acid (10 mmol/l), BGG: bovine gamma globulin (5 w/v %). M, C and BGG were mixed in a 1:1:1 ratio (on a volume basis).

DISCUSSION

To our knowledge, this is the first *in vitro* study to have demonstrated that serum proteins such as FBS, BSA and BGG are factors involved in the production of rounded M-C crystals that appear similar to the brown melamine

crystals that accumulate in the renal tubules of patients with melamine disorders [9].

Previous studies had suggested that pH was a factor that might affect M-C crystal formation and morphology [11, 12, 16]. Normal human urine contains excess water and electrolytes with a pH range of 4.6–8, and lacks blood and proteins [10]. However, we confirmed in this *in vitro* study that needle-like M-C crystals formed in suspensions containing no proteins, within a pH range of 5.6–8.1 (Table 1), suggesting that pH is not involved in formation of rounded M-C crystals. This study clearly showed that rounded M-C crystals formed in suspensions containing serum proteins such as FBS and BSA. The effect of the urinary protein B2M was less marked. Although the pH of the suspensions became alkaline, it did not seem to affect M-C crystal formation (Table 2 and Fig. 2). The present filtration study also strongly suggested that rounded crystals can be formed in the presence of serum proteins. Although the actual mechanism responsible for this phenomenon still remains unclear, it is possible that proteins might exert a catalyst-like effect on the triple hydrogen bonding of the melamine cyanurate complex, and thus contribute to a change in the crystal morphology [24].

On the basis of existing clinical data and our present findings, we suggest a preliminary model to explain how rounded M-C crystals could form in the nephron. Briefly, proteins at two possible sites, i.e., the glomerulus and renal tubules, might be involved. First, the glomerulus, which is a network of capillaries surrounded by Bowman's capsule, has three major layers (filtration barriers) including podocytes, which are mainly responsible for renal excretion by filtering small molecules such as sugars, electrolytes and small serum proteins, including B2M, through the glomerular capillary wall. [8]. Normally, large molecules are unable to pass through the capillary wall. However, in the process of melamine and/or cyanuric acid filtration, some yet unknown defects might occur in the glomerular barriers, thus leading to leakage of large protein molecules into Bowman's capsule and formation of rounded M-C crystals in the renal tubules. Although a recent medical report has indicated that melamine might cause glomerulosclerosis and proteinuria in humans, its pathogenetic effect in the glomerulus still remains unclear [20]. We need to verify whether or not such glomerular defects could cause a considerable amount of protein leakage that would be sufficient to induce downstream accumulation of the M-C crystals. Another possibility is that altered conditions in the tubule lumen, including proteinuria, could arise through decreased re-absorption and/or unusual secretion of proteins from malfunctioning tubule cells [6, 21]. This might induce morphological transformation of M-C crystals and eventually contribute to their accumulation as urolites.

Although further details of the mechanism involved remain to be clarified, our proposed model for the involvement of proteins somewhere in the nephron seems to provide a potential explanation for the *in vivo* pathogenesis of melamine-induced urolithiasis.

REFERENCES

1. An, L., Li, Z., Yang, Z. and Zhang, T. 2011. Cognitive deficits induced by melamine in rats. *Toxicol. Lett.* **206**: 276–280. [[Medline](#)] [[CrossRef](#)]
2. Anonymous. 1973. Anemia. p. 16. In: The Merck Veterinary Manual, 4th ed., Blood, Lymphatic and Cardiovascular Systems (Siegmund, O. H., Fraser, C. M., Archibald, J., Blood, D. C., Henderson, J. A., Howell, D. G. and Kitchell, R. L. eds.), Merck & CO., Inc., New Jersey.
3. Bizzari, S. and Yokose, K. 2008. Melamine. In: Chemical Economics Handbook. Menlo Park. CA. SRI Consulting. Inc. [cited 2008 November 17]. Available from <http://www.sriconsulting.com/CEH/Public/Reports/673.3000/>.
4. Brown, C. A., Jeong, K. S., Poppenga, R. H., Puschner, B., Miller, D. M., Ellis, A. E., Kang, K. I., Sum, S., Cistola, A. M. and Brown, S. A. 2007. Outbreaks of renal failure associated with melamine and cyanuric acid in dogs and cats in 2004–2007. *J. Vet. Diagn. Invest.* **19**: 525–531. [[Medline](#)] [[CrossRef](#)]
5. Chen, K. C., Liao, C. W., Chou, C. C., Chang, S. C., Wu, J. H., Zen, J. M., Chen, Y. T. and Liao, J. W. 2009. Evaluation of Sub-chronic Toxicity of pet Food Contaminated with Melamine and Cyanuric Acid in Rats. *Toxicol. Pathol.* **37**: 959–968. [[Medline](#)] [[CrossRef](#)]
6. Dalal, R. P. and Goldfarb, D. S. 2011. Melamine-related kidney stones and renal toxicity. *Nat. Rev. Nephrol.* **7**: 267–274. [[Medline](#)] [[CrossRef](#)]
7. Dobson, R. L. M., Motlagh, S., Quijano, M., Cambron, R. T., Baker, T. R., Pullen, A. M., Regg, B. T., Bigalow-Kern, A. S., Vennard, T., Fix, A., Reimschuessel, R., Overmann, G., Shan, Y. and Daston, G. P. 2008. Identification and characterization of toxicity of contaminants in pet food leading to an outbreak of renal toxicity in cats and dogs. *Toxicol. Sci.* **106**: 251–262. [[Medline](#)] [[CrossRef](#)]
8. Karnovsky, M. J. and Ainsworth, S. K. 1972. The structural basis of glomerular filtration. *Adv. Nephrol. Necker Hosp.* **2**: 35–60. [[Medline](#)]
9. Kobayashi, T., Okada, A., Fujii, Y., Niimi, K., Hamamoto, S., Yasui, T., Tozawa, K. and Kohri, K. 2010. The mechanism of renal stone formation and renal failure induced by administration of melamine and cyanuric acid. *Urol. Res.* **38**: 117–125. [[Medline](#)] [[CrossRef](#)]
10. Lauridsen, M., Hansen, S. H., Jaroszewski, J. W. and Cornett, C. 2007. Human urine as test material in ¹H NMR-based metabolomics: recommendations for sample preparation and storage. *Anal. Chem.* **79**: 1181–1186. [[Medline](#)] [[CrossRef](#)]
11. Lu, X., Wang, J., Cao, X., Li, M., Xiao, C., Yasui, T. and Gao, B. 2011. Gender and urinary pH affect melamine-associated kidney stone formation risk. *Urol. Ann. May-Aug* **3**: 71–74.
12. Martín Hernández, E., López, C. A., Calatayud, G. A. and Herrera, M. A. G. 2001. Vesical uric acid lithiasis in a child with renal hypouricemia (in Spanish; Castilian). *An. Esp. Pediatr.* **55**: 273–276. [[Medline](#)]
13. NTP. 1983. Carcinogenesis bioassay of melamine (CAS No. 108-78-1) in F344/N rats and B6C3F1 mice (feed study). Research Triangle Park, NC, and Bethesda, MD, United States Department of Health and Health Service, Public Health Service, National Institutes of Health, National Toxicology Program (NTP) Technical Report Series No. 245; NTP-81–86; NIH Publication No. 83–2501.
14. OECD. 1999. Screening Information Dataset (SIDS) for isocyanuric acid. CAS No. 108-80-5. Paris, Organization for Economic Co-operation and Development [cited 2010 September

- 21]. Available from <http://www.inchem.org/documents/sids/sids/108805.pdf>.
15. Puschner, B., Poppenga, R. H., Lowenstine, L. J., Filigenzi, M. S. and Pesavento, P. A. 2007. Assessment of melamine and cyanuric acid toxicity in cats. *J. Vet. Diagn. Invest.* **19**: 616–624. [Medline] [CrossRef]
 16. Quaggin, S. E. and Kreidberg, J. A. 2008. Development of renal glomerulus: good neighbors and good fences. *Development* **135**: 609–620. [Medline] [CrossRef]
 17. Reimschuessel, R., Evans, E. R., Stine, C. B., Hasbrouck, N., Mayer, T. D., Nochetto, C. and Gieseke, C. M. 2010. Renal crystal formation after combined or sequential oral administration of melamine and cyanuric acid. *Food Chem. Toxicol.* **48**: 2898–2906. [Medline] [CrossRef]
 18. Report of a WHO Expert Meeting. 2009. Toxicological and Health Aspect of Melamine and Cyanuric acid [cited 2012 February 11]. Available from http://www.who.int/foodsafety/publications/chem/Melamine_report09.pdf.
 19. Suchy, P., Strakova, E., Herzig, I., Stana, J., Kalusova, R. and Pospichalova, M. 2009. Toxicological risk of melamine and cyanuric acid in food and feed. *Interdisc. Toxicol* **2**: 55–59.
 20. Sun, N., Shen, Y. and He, L. 2010. Histopathological features of the kidney after acute renal failure from melamine. *N. Engl. J. Med.* **362**: 662–664. [Medline] [CrossRef]
 21. Waller, K. V., Ward, K. M., Mahan, J. D. and Wismatt, D. K. 1989. Current concept in proteinuria. *Clin. Chem.* **35**: 755–765. [Medline]
 22. Wang, C., Qin, X., Huang, B., He, F. and Zeng, C. 2010. Hemolysis of human erythrocytes induced by melamine–cyanurate complex. *Biochem. Biophys. Res. Commun.* **402**: 773–777. [Medline] [CrossRef]
 23. World Health Organization. 2008. Melamine-contaminated powdered infant formula in China in 2008 [cited 2012 February 18]. Available from http://www.who.int/csr/don/2008_09_19/en/.
 24. Yagai, S. 2006. Supramolecular complexes of functional chromophores based on multiple hydrogen-bonding interactions. *J. Photochem. Photobiol. Chem.* **7**: 164–182. [CrossRef]
 25. Yang, J., An, L., Yao, Y., Yang, Z. and Zhang, T. 2011. Melamine impairs spatial cognition and hippocampal synaptic plasticity by presynaptic inhibition of glutamatergic transmission in infant rats. *Toxicology* **289**: 167–174. [Medline] [CrossRef]