Review Article

Melamine-contaminated milk formula and its impact on children

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The melamine contaminated milk powder contamination scandal occurred in China in 2008. Its main consequences so far have been urinary stone formation in children with associated renal damage and increased child mortality. Eight years have passed, but food safety issues still remain of concern in the daily lives of millions of Chinese. Vigilance is required to ensure no recurrence of such food safety problems. Ongoing studies focus on the early detection of food industry malpractice, mechanisms whereby these toxic substances induce disease and how its advent may be prevented and better managed. Melamine undergoes renal excretion, but is metabolized slowly and excreted largely unchanged in the urine. Urinary melamine measurement may provide a rapid and inexpensive way to identify exposure to melamine adulterated food items. Although most patients with melamine-related urinary stones (MUS) have been responsive to conservative treatment, longer time follow-up is needed to assess chronic effects. Aside from MUS, melamine is a recognized carcinogen and can induce urinary tract tumours. Very little is known about the effects of excessive exposure to melamine contaminated milk powder in infants on growth, adolescent and adult health, although short-term effects have become apparent during the scandal.

Key Words: melamine, mechanism, clinical features, detection, follow up

INTRODUCTION

In October of 2008, the melamine contaminated milk powder scandal broke in China. Melamine had been added illegally to milk to increase, deceptively, its presumed protein concentration, as assessed by nitrogen measurement, and so as to appear to meet the national standard for milk protein in China. At least 294,000 children were afflicted by this deception. Of these, about 52,000 infants were hospitalized on account of melamine-related urinary stones (MUS) and at least 6 died from the illegal practice.[1] Eight years have passed, but food safety is a daily concern for millions of Chinese people. The lessons learned from the melamine scandal should help to reduce future risk and minimise secondary organ damage in children and others who have been exposed.

All affected infants with MUS had a history of consuming formula milk powder manufactured predominantly by a Chinese company named Sanlu. The concentration of melamine in milk was as high as 2,563 mg/kg in some samples from this company. Compared to the melamine contamination of pet food, the level of cyanuric acid (which increases the risk of melamine nephrotoxicity) was minimal in infant milk powder.[2] Nationwide screening soon found that over 20 dairy companies used a similarly illegal approach to mislead governmental quality control. MUS cases were also found in Hong Kong although the concentration of melamine was much lower in the infant formulas in the Hong Kong market than in mainland China (68 mg/kg vs 2,563 mg/kg). The prevalence of renal calculi in children who consumed melamine tainted milk products was also much lower in Hong Kong than in mainland China (0.03% vs 2.58%).[1,3]

Numerous cases were reported once the scandal became known, and the short term response to the treatment

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of MUS has seemed satisfactory. However, the long-term effect of melamine contaminated formulas on child development is unclear beyond the limited follow-up studies up to 4 years.[4] Under-height and under-weight infants are more common among MUS-exposed than normal children ($p<0.0001$). Children with MUS also have lesser head circumferences and are shorter in the 2-4 year follow-up period after exposure.[5] Although melamine milk powder pollution seems effectively controlled, food safety problems still exist. For example, three rice mills in central China's Hunan Province have been investigated after rice they produced was found to be contaminated with cadmium in 2013.[6] Therefore, monitoring and surveillance have become crucial.

In this review, we describe the pathogenesis of melamine toxicity in infants, its clinical manifestations, management, and long-term follow up with particular reference to melamine-related stones and technologies available for melamine detection.

PATHOGENESIS OF MELAMINE INDUCING KIDNEY DAMAGE

Melamine is a triazine compound with a molecular formula of C3H6N6 and is used in manufacturing household utensils and ornaments.[7] Although a component of flame retardants, glues and plastics, melamine is prohibited from use in any human or animal food preparation. Nevertheless, it was used as an illegal additive as indicated above.[8] Melamine can be present at low levels in food due to its legal use in food packing materials, and in feedstuffs for poultry and livestock. It can also be a metabolite of the pesticide cyromazine.[9] Melamine is harmful for infants who, as a consequence, suffer retarded growth, urinary stone formation and renal failure. This toxicity in early life raises the possibility of uncertain longer-term consequences.[10] Information is scant in regard to the tolerable daily intake (TDI) for melamine which is currently set at 0.2 mg/kg per day (WHO-recommendation).[11] However, there is evidence for renal damage at low exposure in adults.[12] There are risks for neoplastic disease, especially of the renal tract, based on animal experimental studies.[13] Experimental evidence shows that dietary melamine induces urinary tract tumors even at low concentration. Therefore, there may be an increased risk for urinary tumors in adult life. Thus, lifelong screening for urinary tumors in the children who have been exposed to melamine contaminated milk powder may be necessary.

Renal toxicity

Melamine and cyanuric acid (MCA) are well-established nephrotoxins, evidenced by several outbreaks of melamine poisoning in animals.[14] In 2004, renal failure occurred in dogs following consumption of a pet food in several Southeast Asian countries.[14] Melamine was implicated as the source of the problem.[15] In 2007, the Food and Drug Administration (FDA) in the USA recalled more than 100 potentially contaminated pet products after pets developed urinary crystals, with associated animal deaths.[14]

However, the exact mechanism of melamine toxicity remains unclear.[16,17] Melamine has a low direct cytotoxicity in vitro when tested as a single agent in kidney epithelial cell lines derived from cats and dogs.[2] Melamine activates nicotinamide adenine dinucleotide phosphate-oxidase (NOX) including NOX1, NOX2 and NOX4, therefore increasing ROS production. Kuo et al found that melamine increased inflammation and oxidative stress via activation of NF-κB/COX-2 and NOX/ROS pathways.[18] Their study also revealed a critical role of NOX in melamine-induced ROS production, which suggests that there might be a clinical use of NOX inhibitors to alleviate melamine toxicity. Several lines of evidence demonstrate that the activation of the NF-κB signaling cascade upregulates the expression of the subunits of NOX in different cell lines.[19,20] Melamine can activate NOX by activating the NF-κB pathway causing the production of ROS, and ROS in turn can stimulate the NF-κB pathway. Through such a positive feedback inflammation is generated and perpetuated.[21] Thus, the cytotoxicity of melamine in normal rat kidney cell lines may result from excessive generation of ROS. Both oxidative stress and inflammation could be responsible for melamine toxicity. Whether these mechanisms might account for melamine-induced renal injury in humans has not been determined. In an in silico study, Ma et al found that four target proteins (glutathione peroxidase 1, beta-hexosaminidase subunit beta, L-lactate dehydrogenase and lysozyme C) may be involved in the nephrotoxicity induced by melamine in addition to kidney crystal formation.[22] Further, the toxicities of melamine and cyanuric acid might also result from a break down in redox balance, perturbing arginine and proline metabolism and disturbing energy homeostasis.

Stone formation

The mortality rates of infants with melamine-related disease are relatively low, but the incidence of stone formation is high (2.58% in mainland China; 0.03% in Hong Kong) (Figure 1). The dose of melamine lethal for 50% of treated rats is approximately 3,200 mg/kg.[23] The half-life of melamine ranges from 2.7 h to 4.9 h in rats,[24] and is about 4 h in pigs.[25] The kidney is the major target organ of melamine toxicity because of its rapid renal clearance.

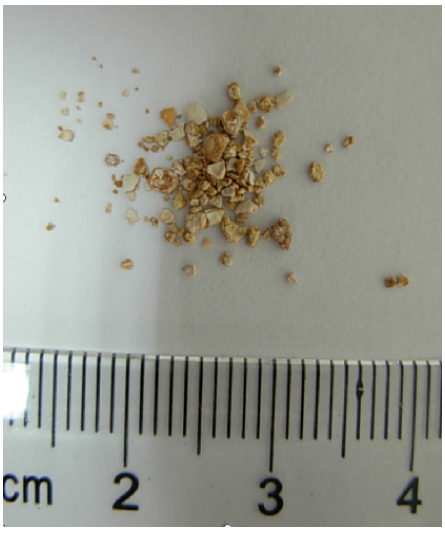

Figure 1. Melamine-related urinary stones (MUS) from Chinese children.

Melamine is barely metabolized by mammals and is excreted in the urine in its original form.[26] Animal postmortems reveal crystalline deposits along with hemorrhagic tubular damage and cystitis.[27] Chen and colleagues established a rat model of MCA induced renal toxicity and showed that crystals were formed in both the proximal and distal tubules. Early acute intoxication features initial proximal tubular cell injury, with subsequent blockage of the distal tubules with MCA-crystals.[28]

Analyses of kidney stones from Chinese infants indicate that the stones are composed of melamine and uric acid,[29] postulated to form a crystalline lattice structure with an equimolar ratio of melamine and uric acid.[30] However, the molar ratio of uric acid to melamine in stones isolated from infants with MUS has ranged from 1:2 to 2.1:1.[31] This means that the structure of melamine–uric acid stones may comprise heterogeneous mixtures of uric acid crystals and melamine–uric acid complexes. Histological studies of infant renal biopsy histology demonstrates melamine-related toxic effects which may in part result from retrograde nephropathy after melamine precipitation in the lower urinary tract.[26,32]

Environmental carcinogens may be excreted in the urine, as is melamine. Dietary melamine can induce urinary tract tumor (UTT) and stone formation in animals even at low concentrations. However, the link between chronic inflammatory urolithiasis and urothelial carcinogenesis is not clear. Melamine induces nephrolithiasis, with associated chronic inflammation. Inflammation may or may not predispose to urinary tumor development. In rodent models, melamine ingestion induces the constellation of nephrolithiasis, chronic inflammation, dysplasia, and in a certain percentage, urothelial carcinomas. Vara Messler et al (2012) proposed that there may be an increased risk for urinary tumors in adult life in children who have suffered from MUS.[33] Clearly, more extensive studies are warranted on urothelial biology, the carcinogenic potential of melamine and the related epidemiology. A preventive diet with polyunsaturated fatty acid supplementation has been recommended on the somewhat tenuous basis of rodent studies.[32]

In brief, through an as yet unknown mechanism, melamine may, in a dose-dependent manner, lead to crystal formation with either endogenous uric acid or a structural analogue of melamine, cyanuric acid, in renal tubules, so increasing the potential for acute kidney failure.[9]

CLINICAL FEATURES, DIAGNOSIS AND MANAGEMENT OF MUS

Clinical features and diagnosis

It is difficult to diagnose calculi in children because of their atypical manifestations.[34,35] The most important information is a history of drinking melamine contaminated formula, along with urinary symptoms and then the findings on ultrasound imaging. However, the sensitivity of ultrasound allows reliable detection down to stones of 2 mm diameter, which covers most stones encountered. Clinical symptoms of infants with MUS vary from none to anuria with renal failure. The main clinical manifestations of melamine related bilateral renal calculi are changes in voiding pattern, including frequency/urgency and oliguria/anuria. Thus, particular attention should be paid to the infants with known or suspected ingestion of melamine-contaminated formula milk, with an elevated urinary melamine/creatinine ratio, and with one or more clinical manifestations of nephrolithiasis, renal failure, and urinary tract infection.

Diagnostic evidence for infantile MUS from ultrasound (US) is a particular advantage. Two-dimensional sonography generates critical information regarding size, location, shape, number, edge, echo and rear sound shadows of stones. In addition, it also shows the degree of hydronephrosis, renal parenchymal compression and internal structural change. Doppler ultrasound can be used to detect renal blood flow and urine flow in the renal hilum and ureter to indicate obstruction. In addition, US diagnosis of urolithiasis is extremely sensitive.[36] Because of its high sensitivity, ultrasound and CT are the first options for urolithiasis diagnosis. Combined methods provide detailed information regarding the location of stones and anatomical deformity, which facilitates future treatment.[37] We have shown that the prevalence of sand-like calculus in MUS is lower than for stones with other causalities ($p<0.05$), but no significant difference ($p>0.05$) in the position and number calculi between MUS and other stones.[38]

According to the World Health Organization and the Ministry of Health of China, the diagnostic criteria for MUS include: 1) history of consuming of melamine tainted infant milk formula; 2) the presence of 1 or more clinical manifestations such as unexplained crying (especially when urinating), vomiting and macroscopic or microscopic hematuria (urinary red blood may be normal on morphological evaluation), oliguria or anuria; 3) a parathyroid hormone test (usually normal); 4) ultrasound examination of the urinary tract which demonstrates stones.

Management

First and foremost this is to cease melamine-contaminated infant formula milk powder immediately on diagnosis. MUS management may be conservative alone or involve surgical intervention as well. Conservative therapy will include diuretics, antibiotics and rehydration if the stones are small and the anuric time is short. Although most patients respond to conservative treatment, surgical treatment is needed if the obstruction persists.[39]

Conservative treatment has been effective because in most cases the urinary calculi are small (<10 mm in diameter) and can be easily dissipated by intravenous hydration**,** greater water intake, alkalization of the urine with 5% sodium bicarbonate intravenously and an antispasmodic drug such as anisodamine or atropine.[40] Other measures include correction of water, electrolyte and acid-base imbalances, closely monitoring blood biochemistry, renal morphology (by ultrasound) and renal function.

In a meta-analysis reported in 2013, Wang et al summarized 26 studies involving 2,164 patients with MUS and found that the majority of patients (94.4%) were successfully treated by conservative therapy. Of 2,040 patients, only 5.6% underwent surgical treatment. The pooled recovery rates at 1, 3, 6, and 12 months after diagnosis or treatment initiation were 67.1%, 76.3%, 85.4%, and 92.3%, respectively. Renal abnormalities persisted in about 8% patients at 12-month follow up, indicating the

necessity of long-term follow-up.[39]

If conservative therapy is not effective, and hydronephrosis and/or kidney failure supervene and persist, where haemodialysis and peritoneal dialysis are unavailable, surgical methods will be considered to relieve obstruction. However, stones can be removed by various methods including cystoscopic retrograde intubation by catheter into the renal pelvis (with perfusion using alkaline fluid), percutaneous kidney drainage, renal lithotripsy or open surgery.[40] Sun et al reported that patients with urinary calculus complicated by acute renal failure should be treated with dialysis or medication to correct electrolyte disturbance, in particular, hyperkalemia. Thereafter, and as soon as possible, the obstruction may be relieved by conservative and surgical methods.[30] The extracorporeal shock wave lithotripter (ESWL) has few indications in infancy, and where the stones are loose and mainly composed of urates. However, in the study of Jia et al, ESWL with low energy could effectively disintegrate MUS and is suitable for treating single MUS in infants and young children.[41] Yan et al assessed the safety and efficacy of minimally invasive percutaneous nephrolithotomy (mini-PCNL) in preschool children with kidney calculi and found that mini-PCNL was an effective treatment for pediatric kidney stones refractory to ESWL, including stones induced by melamine-contaminated milk powder.[42]

We retrospectively analyzed clinical data of 50 children suffering from bilateral renal calculi in 2010.[43] All patients immediately stopped consumption of melamine tainted infant milk formula and began conservative treatment according to the above-outlined procedures. After a short period of hospital treatment (mean±SE stay 8.1±0.7 days), most cases had a good response to conservative management. There was complete dissolution of stones in 21 and partial dissolution in 29 patients. One month later, 12 of these children became stone-free. Hemodialysis was performed in 8 patients with renal failure who did not respond to conservative management after 1 to 2 days. Of the 9 patients with bilateral obstruction and renal failure, only 1required ureteral catheterization for 1 week to drain the renal pelvis due to lack of improvement following 4 hemodialysis sessions. In this patient the stone size decreased and the stone was finally expelled.

In infants with hydronephrosis, it is important to differentiate congenital hydronephrosis induced by congenital pelvic-ureteral junction obstruction (HNUPJO) from those induced by MUS (HNMS) before determining therapeutic plans.[44] The mainstream management for congenital hydronephrosis due to the ureteric-pelvic junction obstruction in infants is close observation or surgical operation. By contrast, infants with MUS complicated with hydronephrosis mainly respond to conservative management.

LONG-TERM EFFECTS OF MELAMINE TAINTED FORMULA ON INFANTS

Although more than half of MUS cases recover after a short hospital stay, many infants and young children still have residual stones at the time of discharge.[45,46] After as long as 4 years follow up, the long-term effects of melamine containment of formula on infants remain unclear.[4] However, this length of follow-up has provided clear evidence that melamine affects child development. Eighty-one children with MUS were recruited in this 4 year follow up study.[4] Of 45 cases with MUS treated conservatively after discharge, stones completely disappeared in 34 cases, partially dissolved in 6 cases, increased in size in 1 patient, and remained unchanged in 4 cases. Of 25 cases with hydronephrosis, 20 completely resolved, 2 reduced in size, and 3 were unchanged. The prevalence of under-height and under-weight infants were significantly higher in those with melamine-related urinary stones compared to controls ($p<0.05$). Zou et al also conducted a descriptive longitudinal study over 2 years in 240 children with MUS in Hangzhou, China. They showed that melamine might damage both the renal glomerulus and tubule with the predominant lesion being urolithiasis. The urolithiasis might persist for over 2 years and cause irreversible damage.[47]

Three possible explanations may hold for the higher prevalence of under-height and under-weight infants with MUS. Firstly, children with MUS may have a poor appetite and inadequate diet in general.[48] Secondly, children with MUS may stay out of the sunlight and be vitamin D deficient. Thirdly, melamine, in addition to its nephrotoxicity, may interfere with the normal metabolism of glucose, protein and nitrogen in the liver.[49] Similarly, another study reported significantly lower body weight and head circumference in melamine-exposed children with calculi than in children without calculi at the time of diagnosis.[50] As indicated, thus far, the longest follow up time has been 4 years, and at that time, the children with severe MUS have malnutrition, as indicated by a lower body height and a smaller head circumference. Such children require long term follow up.

DETECTION OF MELAMINE IN FOOD AND URINE

Melamine detection in food and biologic samples has become of considerable interest since the events surrounding milk powder contamination.[51,52,53] Melamine detection methods include some which are highly selective as well as rapid screening methods. Liquid and gas chromatographic methods conjugated with mass detection are the most powerful methods to confirm the presence of melamine and related compounds. Rapid screening methods like enzyme-linked immunosorbent assay (ELISA) are faster and less expensive. Melamine can decrease the stability of citrate-stabilized gold nanoparticles (AuNPs), and cause dramatic and visible color changes. Thus, a rapid, simple, and sensitive colorimetric detection method for melamine has been proposed based on pyridine-3-boronic acid modified AuNPs.[54,55] This method can detect melamine in milk and milk-based products. Compared to the traditional fluorophore method, it is less sensitive to the changes in the PH value.[56] Chitosan stabilized gold nanoparticles have also been used.[57] For rapid melamine detection, surface-enhanced Raman spectroscopy (SERS) is a reliable tool.[58,59] Li et al (2015) found that immunological separation integrated with SERS may separate and detect trace levels of melamine in milk within 20 mins.[60] A large number of milk samples can be screened using this method with high throughput. There is also a high-

performance liquid chromatography–based method to detect melamine.[61] Yet again, a capillary electrochromatographic technique with a mass spectrometric detection–based method has been described for melamine detection.[62] Even so, technical challenges still exist in melamine analysis in regard to matrix effects, solubility of the melamine-cyanuric acid complex, and background contamination.

The WHO has recommended that the TDI for melamine be 0.2 mg/kg body weight or less. Meanwhile, maximum residue levels (MRL) for melamine have also been set worldwide. In China and the US, the MRL for infant formula has been set at 1.0 mg/kg (1 ppm) and at 2.5 mg/kg (2.5 ppm) for milk and other milk products, while in Europe, the Food Safety Authority has set the limit to 2.5 mg/kg (2.5 ppm) for all products containing greater than 15% milk.[11,63]

To the best of our knowledge, routine clinical chemistry laboratories are generally not equipped to evaluate melamine in biological samples. Melamine is metabolized at a slow rate, and in rats, the large majority is excreted in the urine unchanged.[64] Similarly, Kong et al found that, with melamine adulteration of food, it is also excreted in the urine of exposed individuals in an unchanged form.[65] Therefore, it is clinically important to have available a simple test that can reliably detect urinary melamine with an acceptable sensitivity, specificity, precision, and accuracy in the routine clinical chemistry laboratory.[66]

LESSONS FROM THE MELAMINE-TAINTED MILK POWDER INCIDENT

The melamine contaminated milk powder events have generated much information about the recognition, mechanisms and management of melamine toxicity. It has been demonstrated that the simultaneous exposure to both melamine and cyanuric acid in livestock, fish, pets and laboratory animals is more toxic than to melamine or cyanuric acid alone.[9] Illegal pet food adulterations with melamine and cyanuric acid caused melamine-cyanuric acid crystals, kidney damage and deaths of cats and dogs in Western countries. Similarly, adulteration of milk with melamine has resulted in melamine-uric acid stones, hospitalisation and deaths of children in China. Following these incidents, the TDI for melamine was re-evaluated by the US Food and Drug Administration, the WHO and the Scientific Panel on Contaminants in the Food Chain of the European Food Safety Authority (EFSA).[9] The WHO and the EFSA have established a TDI of 0.2 mg/kg/day for melamine and 1.5 mg/kg/day for cyanuric acid after the Chinese milk adulteration incidents.[11]

The nature and pathology of crystal formation between melamine and other structural analogs, namely ammelide and ammeline, is not well understood. More studies of adulteration practices and risk assessment for melamine and its structural analogues, for both animal and human health at 'background' levels of melamine from various sources and for its structural analogues used in animal feed are required.

Food safety is critical not only for immediate and long-term health, but also because of its importance to sustainable development, national economies, trade and tourism. With globalization, unsafe food poses a global threat to practically everyone, but particularly to vulnerable groups like infants, young children, pregnant women, the elderly and those already ill. As the world's population grows, the intensification and industrialization of agriculture and animal production to meet the increasing demand for food creates both opportunities and challenges for food safety.[11] As long as business continues and government regulations are not appropriately followed, the opportunity for recurrence of this type of event remains. Although the Chinese government has strengthened the regulations, incidents of melamine contamination of food, such as with candy, are still being reported.

The Chinese Ministry of agriculture has carried out a seven-consecutive-year raw milk quality monitoring program for melamine and other prohibited additives in which the sampling qualification rate has remained at 100%, while the paddock scale for raw milk protein and milk fat content have been significantly higher than the national standard.[67] Since 2009, agricultural departments nationwide have closely supervised the raw milk industry and cracked down on the illegal addition of substances to raw milk. Although the supervision has focused on raw milk produced for infant formula and a source-tracing monitoring system has been established to guarantee the safety of infant formula, other unsafe foods are still found.

The Chinese government updated the "Food safety law of the people's Republic of China" recently, with implementation on Oct 1st, 2015. This revision of the Food Safety Law aims to establish the strictest legal responsibility of each party so as to realize the shared governance of food safety. China's legislative body is making a determined effort to strengthen the management of food safety to guarantee safety at "the tip of the tongue".

Although the quality of domestic milk has improved, consumers are not reassured and quality concerns are evident in feedback findings. Internet users question the quality of domestic milk powder, and people ask about the use and presence of antibiotics and whether aflatoxin is to be found in domestic milk powder, beside those to do with melamine.[68] There is still a long way to go to guarantee and reassure the community about food safety.

CONCLUSION

The melamine contaminated milk powder events provide lessons for future risk management approaches to food safety through earlier detection, quick response and crisis management, prevention plans and better clinical management protocols. The children affected by melamine stones in the urinary tract require that not only they themselves have long-term follow-up on account of the risk of urinary tract tumors, but also so do their exposed peers in whom lower levels of exposure may yet matter. With the rapid industrialization of developing nations, new environmental toxins may continue to emerge and similar food safety events encountered. For years to come, and always, the prevention and risk management of food contamination must remain a research, health and public policy priority.

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AUTHOR DISCLOSURES

None of the authors have any conflicts of interest associated with this study.

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Review Article

Melamine-contaminated milk formula and its impact on children

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三聚氰胺污染奶粉及其对儿童的影响

2008 年，中国发生了三聚氰胺污染奶粉的丑闻事件。到目前为止，其主要后果是引起众多婴幼儿患泌尿系结石，部分患儿出现肾衰竭，甚至死亡。八年过去了，但食品安全仍是数以万计中国人日常关心的大事。我们仍需警惕类似食品安全事件的发生。未来的研究应着重关注类似食品添加剂的早期检测、发病机制和诊疗技术等。三聚氰胺在人体内通过肾脏代谢，且三聚氰胺代谢速度较慢，大部分以原形经尿液排出。因此，检测尿液中的三聚氰胺可能是一种快速、经济的检测三聚氰胺污染的有效方法。尽管三聚氰胺泌尿系结石患儿经保守治疗后大部分可痊愈，但对这部分患儿的长期随访研究仍十分重要。除了泌尿系结石，三聚氰胺也可能引起泌尿系肿瘤。尽管从三聚氰胺污染奶粉事件中可以看出三聚氰胺对婴幼儿的短期影响，但过多暴露于三聚氰胺污染的奶粉对婴幼儿生长发育、青少年和成年期健康的影响仍需进一步随访研究。

关键词：三聚氰胺、机制、临床特点、检测、随访