Evaluation of the Mode of Action of Melamine-induced Urinary Bladder Tumors in Rats and an Assessment of its Human Relevance

Introduction

Melamine is a non-genotoxic chemical and was shown to induce an increased incidence of urinary bladder tumors in male rats when administered as 4500 ppm of the diet but not as 2250 ppm of the diet (1). It was associated with urothelial hyperplasia and with the formation of urinary tract calculi. Calculi and hyperplasia also occurred in female rats and in both genders of mice, but did not result in an increased incidence of tumors. There is a strong correlation between the formation of the calculi with induction of cytotoxicity of the urothelium, with consequent regenerative hyperplasia and eventual formation of tumors (2). This sequence of events has been well demonstrated in male rats (1-3). Calculi with hyperplasia occurred in mice but without tumors. Although mice tend to be less susceptible to the tumorigenic effects of calculi, such an effect can occur as reported with uracil and other chemicals (4). It clearly involves a threshold. Administration at high doses to rodents (4500 ppm of the diet) produced calculi and consequent urothelial proliferation, but administration at 2250 ppm of the diet did not.

A similar sequence of events regarding crystal formation was demonstrated in the adulteration of pet foods, particularly for dogs and cats (5), with a consequent formation of urinary tract crystals and calculi. In this instance, the adulteration was by a combination of melamine and cyanuric acid, which together formed a hydrogen bonded lattice crystalline network (6). This provided a decrease in the expected solubility of melamine, but was still a high dose effect. A similar episode occurred with the adulteration of infant formula in China leading to formation predominantly of intrarenal crystallization, but with occasional formation of lower urinary tract calculi (7). In all of these instances, the effect has been a threshold response, which is expected on a physical chemical basis for the formation of crystals within the urine. The reason that crystallization occurs in the urinary tract rather than elsewhere in the body is due to the predominant excretion and concentration of melamine in the urine, producing a much higher concentration in urine than in blood.

Urinary tract solid formation is a threshold phenomenon, with a physico-chemically defined dose required to attain crystallization in the urinary tract. If the dose is insufficient to lead to crystallization, there is no cytotoxicity, no regenerative proliferation, and no tumor formation. Tumor formation requires long-term presence of the urinary solids. The concept of crystals and calculi leading to the toxicity and carcinogenicity of several chemicals was first delineated by David Clayson (8) at Health Canada in 1974, based on experiments he was performing at that time on a sulfonamide drug, and research that he had previously performed on the instillation of paraffin wax or cholesterol pellets into the bladders of mice.

Extrapolation from Rodents to Humans

Concern has arisen that the demonstration of crystals and calculi in humans in the Chinese infant formula episode changes the understanding about the potential carcinogenicity of melamine for humans. However, that is not the case. Urinary tract crystals and calculi can be produced in rodents, pets, and humans by a variety of substances, including essential ingredients in our diet (calcium, magnesium) a variety of drugs (acetazolamide, sulfonamides, and HIV protease inhibitors), and a variety of chemicals (9). Many of these substances have produced urinary tract tumors in rodents, especially in rats, so that the evaluation is entirely on the relationship of urinary solids to tumors, rather than on the specific chemicals. This issue has been reviewed by a wide variety of agencies, including the

International
Agency for Research on Cancer, the U.S. Environmental Protection Agency, the European Chemical Agency, European Medicines Agency, Food and Drug Administration, and Health Canada, amongst others. The conclusion has been that crystals and calculi pose a threshold toxicity concerns for humans, but not carcinogenic risk. This is true regardless of the substance that produced the calculus in the first place. For example, calcium-containing salts are the most frequent calculi that form in the human urinary tract. Obviously, calcium is not considered carcinogenic but at sufficiently high doses the extensive regulatory network responsible for calcium homeostasis can lead to formation of calculi in humans. The basis for the species difference in susceptibility to carcinogenesis from urinary solids has not been completely explained, but nevertheless, is a consistent finding. The rat appears to be the most susceptible species, followed by the mouse.

Furthermore, for human exposures to melamine, calculi and urinary tract effects have only received attention in relation to consumption of adulterated baby formula which was the sole source of food for the infants, resulting in consumption of a high daily dose of melamine on a mg per kilogram body weight basis. Such a high dose is necessary for the formation of melamine-containing crystals. Children and adults with numerous sources of food would never attain such a dose, thus, crystal formation would not occur.

Urinary Solids in Humans

There are several reasons why the urinary tract of humans is not susceptible to the carcinogenic effect of urinary tract solids. It ultimately comes down to a variety of anatomic differences between rodents and humans (10). In rodents, calculi can accumulate in the dome of the bladder without complete obstruction of the urinary tract, so that the calculi can remain present in the urinary tract essentially for the lifetime of the animal without complete kidney deterioration. In contrast, in the human urinary tract, calculi may produce complete obstruction of the urinary tract. This is partly due to the vertical nature of human stature so that stones cannot accumulate in the bladder, but also because of specific anatomic factors in humans. Specifically, there are several points along the urinary flow which have significant narrowing of the urine passage so that stones can cause obstruction at these sites. These include the site at which the kidney pelvis narrows to become the ureters, secondly, at the point where the ureters cross the pelvic brim, and thirdly, the traversing of the ureter through the urinary bladder muscular wall. If urinary crystals and small calculi form, they will readily be excreted without obstruction, without damage to the urothelium, and will not accumulate in the urinary bladder. Crystalluria in humans does not have the same effect it can have in rodents where cytotoxicity of the urothelium can occur with consequent regenerative proliferation (11). In contrast, in humans, crystals either have no effect, or they are a predictor of the formation of calculi. By themselves, the crystals do not appear to produce toxicity of the human urothelium. If they accumulate in the renal tubules, then toxicity can occur, which has been most widely studied in the relationship of calcium oxalate crystals forming secondary to the ingestion of large quantities of ethylene glycol (antifreeze) (12). Thus, substances such as sodium saccharin, sodium ascorbate (vitamin C), sodium bicarbonate (baking soda), sodium chloride (table salt), and a variety of other sodium salts can produce crystalluria in rats leading to the formation of bladder tumors, but this does not occur in humans (2, 9). Likewise, a variety of crystals that occur in humans by themselves are not toxic to the urinary tract, such as uric acid, calcium phosphate, calcium oxalate, calcium carbonate, a variety of magnesium salts, and others, unless they form calculi.
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If calculi actually do form in humans and are large enough to obstruct the urinary tract rather than being immediately excreted in the urine, complete obstruction occurs resulting in excruciating pain. When this

happens, the calculi are either spontaneously voided following ingestion of large amounts of liquids, or they are removed ultrasonically or by surgery. This is a major difference between the effects in rodents and humans since the urinary tract tumor formation is dependent on the long term presence of these urinary crystals and calculi in the rodents. In rare circumstances, calculi actually can remain in the human urinary tract for long periods of time, such as in diverticuli or secondary to neurogenic bladder, but these are usually associated with bacterial contamination and cystitis. Cystitis by itself, whether secondary to bacteria or schistosomiasis, leads to a slight increased risk of urinary tract tumors (13). Diverticuli, whether containing a calculus or not, carry an increased risk of bladder tumor formation, presumably secondary to the stagnation of urine in the diverticulum (13). Overall, the weight of the evidence leads to the conclusion that calculi by themselves are not carcinogenic to the human urinary tract. This is true for melamine as well as the wide variety of other substances for which calculi have been produced in humans.

This issue was addressed specifically by an international panel convened by the U.S. EPA and published in a series of articles in Food and Chemical Toxicology in 1995 including an overview by the working group (14). This was followed by a review by IARC which was published in an IARC scientific publication in 1999 (2) leading to the conclusion that urinary tract solids are not relevant to human cancer. This issue was predominantly focused on sodium saccharin and other sodium salts, which form urinary tract crystals and seldom produce calculi, but also addressed the issue overall of urinary tract calculi. This was subsequently applied to a review of a number of substances that produced urinary tract tumors secondary to the formation of urinary solids, including melamine, which was published in the IARC Monograph series, volume 73. In that evaluation, it was concluded that melamine was to be classified as a class III agent, and that the rodent bladder tumors were not predictive of a cancer risk to humans (15).

Another panel that was convened by the U.S. EPA and Health Canada (16), and subsequently expanded by the International Programme on Chemical Safety (IPCS) (17) was convened to develop a mode of action and human relevance framework. Melamine was considered as one of the case studies, and was published in 2003 (16). I was a member of the 1995 EPA panel, the IARC panels, and the mode of action/human relevance panels.

Melamine-adulterated Foods

The lack of potential cancer risk to humans and lack of potential toxicity at low exposure were also considered by a group of six scientists contacted by the U.S. FDA following the pet food contamination episode (18). It turned out that not only had the melamine-adulterated diet been given in pet foods, but had also been incorporated into foods given to pigs, chickens, and fish which became part of human foods. This raised a concern about toxicity for humans. Six scientists were contacted independently, including myself, without telling us who the others were so that each of us could form an independent assessment. It was concluded that if any one of the six scientists had any concern whatsoever regarding the toxicity of this adulterated diet to the human food supply, the FDA would have acted to remove all such foods from the marketplace. The conclusion was that this did not pose a toxic risk for the levels present in human foods. Similarly, following the episode of the infant formula adulterated with melamine, an evaluation was made by the Chinese government. I was contacted by the Chinese government regarding this issue. Although calculi and crystals have persisted in some of these children (raising questions about standard of care since calculi are usually removed), there was no evidence of neoplasia although there continued to be evidence of renal toxicity.

Urinary Calculi and Cancer in Humans

The CLH has presented evidence that urinary calculi may lead to lower urinary tract tumors and also renal cell carcinomas. There are several issues that need to be addressed regarding what is presented.

They discuss the publication of Liu et al., regarding low levels of urinary melamine increasing the risk of urinary calculi in general (20). However, the calculi detected in that study were calcium-containing calculi. In the experimental rodent studies, the pet food contamination event and the infant formula event, the crystals and calculi always were predominantly composed of melamine, usually with uric acid or/and cyanuric acid. Finding low levels of melamine in calculi in the Liu et al., study was most likely due to melamine in the urine being incorporated into a calcium stone, but this possibility has not even been considered by the authors. Today's sophisticated and ultrasensitive, analytical chemistry methods are able to detect these low levels in the calculi. However, these are calcium stones, the melamine is one of many urinary products that could be incorporated into the stone as it forms and be detectible, but not contributing to the actual formation of the stones.

The Liu et al. study does raise a common confounding and bias factor that is present in all studies regarding calculi in patients. Patients with calculi are monitored for recurrence closely. This leads to frequent urinalyses and imaging studies and increased awareness by the patients of urinary tract issues. This increased medical surveillance produces a confounding factor. In the Liu et al. study, the control individuals were randomly selected from subjects receiving routine health screening. The issue of increased surveillance leading to increased detection of urinary tract tumors has been described in diabetes (21). In addition, in the Liu et al. study, there was a higher proportion of cigarette smokers and betel quid users that might have contributed to stone or tumor formation.

CLH also presents several studies that show an increase of urinary tract tumors associated with individuals with stones. All have the issue of confounding related to increased surveillance, which is explicitly stated in the publication by Cheungpasitporn et al. (22). They also indicate that the studies in their meta-analysis, "lacked information regarding potential confounding factors, such as family history of kidney cancer, cigarette smoking, body mass index, dieting and occupational exposure…." That analysis was mostly related to renal cell cancer rather than urothelial cancer. CLH raises renal cell cancer as a possible result of calculi in addition to urothelial cancer. The evidence for such an association is weak, and the report does not present other studies that have not found an effect. Furthermore, no renal cell tumors were observed in the rat or mouse studies, even though the spontaneous evidence of kidney tumors in rats is greater than for the bladder. CLH also describes the inflammation in the kidney of rodents administered melamine as a plausible mode of action. However, that inflammation is due to pyelonephritis or retrograde nephropathy (23), neither of which is associated with increased incidences of renal cell tumors in rodents or humans.

The studies that were referred to in the CLH report regarding calculi and increased risk of urothelial tumors all have the issue of surveillance confounding and bias. Some of the studies control for confounding factors, such as cigarette smoking. However, the issue of bacterial infection is not adequately addressed in these studies if at all. Bacterial cystitis can be intermittent and can be clinically asymptomatic. Asking an individual if they have cystitis is an inadequate method to ascertain urinary tract infection. Urinalysis and cultures are required. Such measures were not performed in these epidemiology studies.

CLH also refers to the increased risk in males compared to females with calculi; most studies showing an effect found so only in males. CLH relates this to the finding of tumors only in male rats, not females. However, in the rat and mouse studies, hyperplasia occurred in males and females. Furthermore, human

urothelial cancers arise much more commonly in males overall, with a ratio usually of about 3:1. Calculi also occur more commonly in males, likely due to the anatomic differences between males and females regarding the ureter and lower urinary tract and also due to urethral narrowing commonly in men secondary to benign prostatic hyperplasia.

In summary, melamine ingested at high levels can form urinary tract crystals and calculi, which can lead to either renal damage or urothelial cytotoxicity with regenerative proliferation and ultimately with continued exposure the formation of bladder tumors in rodents. However, regardless of the substance that produces the calculi, formation of such tumors cannot be extrapolated to humans (2, 15, 19). Thus, despite the unfortunate infant formula adulteration episode in China, the overall assessment remains the same. The formation of calculi in humans poses a toxicity issue at high doses but does not lead to increased tumor incidences in humans. To label a substance as having carcinogenic activity based on calculi is not only not scientifically justified but would raise the specter that several essential dietary components, such as calcium and magnesium as well as common non-essential dietary components, such as cysteine, carbonate, uracil, uric acid, and many others, would also have to be labeled.

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