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## Renal and vascular function in pregnant and neonatal rats exposed to melamine and related compounds

### Key Messages

1. In rats, long-term exposure to melamine impairs renal blood flow and renal vascular function in a dose-dependent manner.
2. Melamine-induced renal vascular dysfunction is caused by enhanced vasoconstriction and reduced vasodilatation owing to the endothelial cell dysfunction of renal arteries.
3. Endothelial dysfunction after melamine treatment is mediated through oxidative stress-dependent activation and up-regulation of cyclooxygenase 2, which produces prostanoids that act on the thromboxane receptor.
4. Transforming growth factor  $\beta$ 1 and bone morphogenic protein 4 contribute to renal fibrosis induced by oral administration of melamine.
5. Exposure to melamine during pregnancy and lactation exaggerates renal vascular dysfunction in the offspring.

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許多嬰兒因長期服食含有三聚氰胺的奶製品導致腎臟中毒和腎結石。這些引起患兒腎功能出現病理變化的機制仍然不清楚。為了解含三聚氰胺及其代謝產物的奶類製品對於健康的影響，我們試圖通過動物研究來解答以下幾個相互關聯的問題：（1）長期每日攝取低劑量三聚氰胺或其代謝產物三聚氰酸會否損傷血管及腎功能。如果可以，三聚氰酸有否在三聚氰胺所造成的損傷中起一定的作用？（2）如果單獨攝取三聚氰胺或三聚氰酸不能損傷腎功能，那麼同時攝取二者會否導致腎臟損傷？（3）孕婦長期攝取三聚氰胺和/或三聚氰酸會否導致出生後胎兒腎功能障礙？因此本研究旨在闡明：（1）嬰兒時期長期低劑量攝取三聚氰胺或三聚氰酸導致成年後的血管功能及腎功能障礙；（2）通過應用血管功能學方法檢測離體腎動脈活性，應用核磁共振檢測腎臟血流量，分析炎症因子及纖維化因子的表達，免疫組織化學以及測量血漿和尿液中三聚氰胺的含量這些方法綜合研究孕鼠長期攝取三聚氰胺或三聚氰酸導致胎兒出生後出現腎功能障礙。本研究提出了三聚氰胺損傷腎臟的新機制：三聚氰胺顯著降低腎臟血流量，損傷腎臟血管功能並且增加腎血管和腎臟炎症因子，轉化生長因子  $\beta$ 1，骨形態發生蛋白4和環氧合酶2的含量。三聚氰胺同時能導致腎臟纖維化和炎症。本研究也為三聚氰胺及其代謝產物如何導致乳鼠腎功能障礙以及胎鼠長期攝取三聚氰胺及其代謝產物對其出生後腎臟功能有何影響提供了部分科學依據，支持了最近觀察到的嬰兒長期攝取含三聚氰胺及其代謝產物的奶製品會導致腎結石及急性腎衰竭這一臨床症狀。

On 11 September 2008, news media reported that thousands of infants and children in Mainland China had suffered from kidney stones and kidney failure and were hospitalised after consuming milk products contaminated with melamine. Excessive exposure to melamine caused the formation of kidney stones and thus severe obstruction of the urinary tract. Four infants lost their lives because of acute renal failure associated with melamine intake. This news caused a widespread public outcry and health scare worldwide. Melamine (1,3,5-triazine-2,4,6-triamine) derived from urea is rich of nitrogen, which was deliberately added to milk products to boost their protein contents on standardised tests that use nitrogen content as a surrogate for protein.

There were few reports on the toxicity of melamine in humans before the outbreak of the melamine incident. Limited research showed that in rats, the toxic dose causing 50% of exposed animals to die was 3.1 g/kg of melamine and 7.7 g/kg of cyanuric acid. The United States Food and Drug Administration has adopted tighter recommendations on the tolerable daily intake (TDI) for melamine, which reduced from 0.63 mg/kg to the present 0.063 mg/kg, as infants may be more susceptible than adults. In addition, both the European Food Safety Authority and the World Health Organization set the TDI for melamine at 0.2 mg/kg body weight/day. The government of the Hong Kong Special Administrative Region has also promptly set a legal limit for melamine in food. The amended Harmful Substance in Food Regulation has limited the level of melamine and related analogues to <1 mg/kg milk and food intended for consumption principally for children under the age of 36 months and by pregnant or lactating women.

There are limited studies examining the effects of melamine and its analogue cyanuric acid in experimental animals (mice, rats, cats, and dogs). Dietary exposure to melamine was found to induce calculi formation, inflammatory responses, and hyperplasia in the urinary bladder. In dogs, melamine crystalluria was also observed. Renal toxicity of melamine was reported in a study involving

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chronic feeding of melamine to female rats, in which there was dose-related accumulation of calculi in the proximal tubules and chronic inflammation of the kidney. Several subchronic oral toxicity studies revealed that cyanuric acid caused renal injury (such as necrosis or hyperplasia of the tubular epithelium, neutrophilic infiltration, mineralisation, and fibrosis), which are associated with the formation of cyanurate crystals. Furthermore, the outbreak of acute kidney failure in cats and dogs in 2007 was related to co-ingestion of both melamine and cyanuric acid.<sup>1</sup> Both compounds may form a complex of very low solubility called melamine cyanurate, which leads to the formation of kidney stone and eventually causes tubular blockage and renal damage.<sup>2</sup>

The outbreak and consequence of nephrotoxicity and kidney stone in infants previously exposed to melamine-contaminated milk products had caused widespread health panic, particularly in parents and pregnant women. Nevertheless, little was actually known about the mechanism leading to pathophysiological alterations in renal function in affected children. To respond to public concerns about the safety of melamine and its related compounds in dairy products, we examined whether (1) neonatal ingestion of melamine causes impaired renal and vascular function in adult rats, and (2) ingestion of melamine during pregnancy affects the renal function of their offspring.

In adult male Sprague Dawley rats, oral ingestion of melamine in drinking water at three dosages (60, 300, and 600 mg/kg body weight/day) for 3 months did not affect body weight or systolic blood pressure, as measured by the tail-cuff method. Melamine consumption at medium and high dose markedly increased its levels in plasma and kidney tissues. Renal cortical blood flow (as measured by magnetic resonance imaging using a 3T clinical whole-body imaging system) diminished after melamine exposure. Melamine ingestion progressively impaired endothelium-dependent relaxations in the isolated rat renal arteries revealed by microvessel myography. In the presence of each of the following drugs: cyclooxygenase-2 inhibitor NS398, the thromboxane prostanoid receptor antagonist S18886, and the reactive oxygen species scavenger tiron plus DETCA, even after medium and high doses of melamine ingestion, such endothelium-dependent relaxation was significantly improved. Melamine ingestion also increased (in a dose-dependent manner) fibronectin accumulation in glomeruli, which was revealed by immunohistochemical staining and indicated unfavourable remodelling of the kidneys and renal arteries. Increased expression of fibronectin was confirmed by Western blotting results in both kidneys and renal arteries. In addition, the expression of several pro-inflammatory biomarker proteins (such as transforming growth factor- $\beta$ 1, bone morphogenic protein-4, and cyclooxygenase-2) also appeared up-regulated in the kidneys and renal arteries of melamine-treated rats.

In 2-month-old female rats treated with melamine

for 2 weeks before mating, their offspring were then given melamine or its vehicle for another 3 months. The male offspring born from high-melamine-dose-exposed mothers attenuated endothelium-dependent relaxation and exaggerated endothelium-dependent contractions in the presence of nitric oxide synthase inhibitor in the renal arteries, as compared to those received the vehicle control.

Findings of the present study on rats regarding the renal and vascular toxicity of melamine ingestion include (1) short-term exposure to melamine and cyanuric acid alone did not cause mortality; (2) short-term exposure to melamine and cyanuric acid combined led to significant reduction of renal blood flow, visible crystal formation in the renal cortex and medulla, and deaths; (3) long-term ingestion of melamine (600 mg/kg/day) impaired function in intralobar renal arteries with an internal diameter of about 200  $\mu$ m, through activation of thromboxane prostanoid receptors, indicating possible inflammatory events leading to the production of cyclooxygenase-derived prostaglandins in the renal blood vessels; (4) renal fibrosis developed in melamine-treated rats; and (5) melamine and cyanuric acid accumulated in circulating blood and the kidneys, as revealed by mass spectrometry. These results provide useful information on the pathophysiology of events related to melamine exposure and toxicity.

The present study also showed that the oral administration of high doses of melamine or cyanuric acid alone did not induce acute toxicity in adult rats even though there was crystal formation in the renal medulla. By contrast, long-term exposure to melamine for 3 months did reduce renal blood flow and impaired renal vascular function. Although the health impact of melamine on adults is not fully understood, the concentration of urinary melamine is positively correlated with the risk of kidney stones.<sup>3</sup> In addition, the combination of melamine and cyanuric acid resulted in severe stone formation and renal toxicity, causing death within a few days, because melamine stones mainly affect renal tubules. In rats, the toxic effects of combined exposure to melamine and cyanuric acid are more severe.

Vascular function of the renal arteries affects renal function through the regulation of renal blood perfusion as well as glomerular filtration. Histologically, the kidney disease, the presence of melamine stones, and an increase of serum creatinine level (indicating impaired renal function) are indicative of the renal toxicity of melamine.<sup>1</sup> Melamine ingestion progressively impairs endothelial cell function, and augments endothelium-dependent contractions in intralobar renal arteries. These harmful effects may well contribute to the reduction of renal blood perfusion. The possible underlying mechanisms may involve increased expression and activity of pro-inflammatory signalling molecules (reflected by the increased expression of transforming growth factor- $\beta$ 1, bone morphogenic protein-4, cyclooxygenase-2, and fibronectin). These

modulators could become therapeutic targets in the fight against melamine-induced nephropathy.

In the present study, exposure of rat mothers to melamine exaggerated melamine-induced renovascular dysfunction in their offspring suggested that melamine was transferred maternally through gestation or lactation. Previous studies also validated the possibility of transfer through the placenta and milk.<sup>4</sup> Taken together, existing evidence definitely raises health concerns about melamine-contaminated food for mothers.

The present study also provided useful experimental results regarding the harmful effects of ingesting melamine, cyanuric acid, or both on the renal function of neonatal/postnatal rats. We showed that melamine and its related compounds dose-dependently impaired renal and vascular function. This was accompanied with the development of interstitial fibrosis and eventual renal damage, through activation of inflammatory pathways or overproduction of reactive oxygen species, followed by decreased bioavailability of nitric oxide. The present study also provided better understanding of the safety profile of long-term exposure of neonatal or pregnant rats to low levels of melamine and related compounds on renal and vascular function in adulthood or in their offspring.

Therapeutic interventional experiments are warranted to provide scientific support for the use of relevant drugs to alleviate the renal toxicity associated with melamine ingestion. Although no effective means are available to

dissolve these kidney stones, oral potassium citrate or sodium bicarbonate are clinically used to alkalinise the urine to dissolve uric acid stones. Rats with tubular crystallisation can be treated with either potassium citrate or sodium bicarbonate, with monitoring of the size and location of crystals and renal function. Moreover, the calcium level in urine can be assessed. If ingestion of melamine and related compounds causes hypercalciuria, diuretics such as thiazides may have the potential to lower urine calcium levels.

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