Melamine toxicity in rat foetuses and infants

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背景：三聚氰胺在胎兒和幼兒的毒性不完全清楚。
主旨：研究三聚氰胺在大鼠胎兒和幼兒的藥物動力、效力及致畸作用。
方法和對象：三聚氰胺及其衍生物氰尿酸在大鼠胎兒和幼兒的毒性實驗。
實驗：妊娠、哺乳及產後大鼠給予三聚氰胺及氰尿酸，並取集胎兒和幼兒標本。
研究項目：三聚氰胺及氰尿酸在大鼠胎兒和幼兒的生物利用、母胎和母乳的轉遞及胎幼兒的分佈和積累、胚胎致畸、胎兒急性中毒及幼兒慢性中毒，以確定在發育及成長的安全性。
結果：三聚氰胺及氰尿酸可通過乳腺及胎盤從母親傳送到幼兒、胎兒及羊水。其動力學特性在妊娠期母體與產後幼兒相反。在妊娠末期胎兒腎臟及產後　周幼兒腎臟的生物利用度最高。胎兒半致死劑量為300毫克/千克，幼兒半中毒劑量為80毫克每千克，每日可容忍攝入量少於0.02毫克/千克。母親攝取三聚氰胺可引起體重及產子量降低，流產及圍產期亡率升高，但不會致畸。
結論：本實驗確定三聚氰胺及氰尿酸經母胎與母乳傳送，主要在未成熟的胎兒及幼兒腎臟分佈和積累。母親接觸可引起不良妊娠和圍產後果。
影響：本研究明確三聚氰胺在大鼠胎兒和幼兒的毒性，並提供有用的產前和產後藥理和毒理數據。

Key Messages

1. Melamine and cyanuric acid can transfer to mammary glands and pass through the placenta to foetuses and amniotic fluid.
2. In rats, pharmacokinetic profiles of maternal samples during gestation and of infant samples during the postnatal period were reversed.
3. High bioavailability was identified in foetal kidneys at late gestation and in infant kidneys at early postnatal period.
4. The foetal LD_{50} was defined as 300 mg/kg, whereas infant IC_{50} as <80 mg/kg and the tolerable daily intake as <0.02 mg/kg.
5. Maternal exposure resulted in no congenital malformation but decreased maternal weight gain and litter size as well as increased pregnancy loss and perinatal deaths.

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Introduction

In 2004, melamine-tainted pet food resulted in numerous incidents of acute renal failure in dogs and cats.[1] In 2008, melamine-adulterated milk powder for infants was discovered in China.[2] The toxicity of melamine has raised concerns for public health in China and other countries. Safety data regarding melamine in pregnant and lactating women are limited. When melamine-contaminated food is consumed during pregnancy and lactation, foetuses and infants may become sensitive to the direct action or to environmental changes produced by chemicals or drugs,[3] and more vulnerable to the toxic effects of melamine. It is unknown whether high levels of melamine can pass through the placenta to the developing foetus, and whether melamine has any toxicity on embryo-foetal development and prenatal and postnatal growth. This study aimed to identify melamine and cyanuric acid toxicity in foetuses and infants during pregnancy and lactation. The specific objectives were to establish the pharmacokinetics, pharmacodynamics, and teratogenicity of melamine and cyanuric acid with respect to foetal and infant rats in vivo.

Methods

This study was conducted from April 2009 to March 2011. Sprague Dawley rats were used. According to the US Food and Drug Administration Guidelines on Detection of Toxicity to Reproduction for Medicinal Products, three experiments were performed to determine pharmacological profiles and potential effects of melamine and cyanuric acid on foetuses and infants. In part I, pharmacokinetics were studied with respect to the bio-availability of melamine and cyanuric acid by measuring their concentrations in foetal and infant samples. In part II, pharmacodynamics were studied with respect to the foetal and infant toxic dosage of melamine and cyanuric acid. In part III, teratogenic dose for potential developmental toxicity was determined based on congenital malformations following maternal exposure.

In part I, single bolus doses of melamine or cyanuric acid were administered to healthy female rats at different reproductive stages, including early, mid, and late pregnancy, and during lactation, as well as to healthy infants at different stages

of maturation. Samples were collected within 24 hours of administration to characterise pharmacokinetic profiles. In part II, single bolus doses of melamine and cyanuric acid in different concentrations were administered to healthy pregnant rats at different stages of pregnancy to determine acute toxicity to the foetus. In addition, multiple bolus doses of melamine and cyanuric acid in different concentrations were administered daily for 2 weeks to healthy infants at different stages of maturation. In part III, repeated bolus doses of melamine and cyanuric acid were administered daily to healthy pregnant rats (at defined gestational stages) and to lactating rats (at different developmental stages) to detect immediate and latent effects of such exposure.

Melamine and cyanuric acid were measured simultaneously by a liquid chromatography tandem mass spectrometry (LC-MS/MS) method as previously described.[4] The UPLC Waters Xevo TQ System (Waters, Milford, MA, USA) was used to detect the melamine and cyanuric acid accumulation in the samples as previously described.[5] Extraction of tissue homogenate samples was adapted using a previously reported method,[6] whereas extraction from serum and biological fluids entailed direct centrifugation at 16 000 g for 5 minutes. After extraction, clear supernatant was transferred to a new sample vial for direct LC-MS/MS analysis. Calibrator or extracted samples were injected into an ACQUITY UPLC BEH HILIC column. 10 ppb stable isotope-labelled melamine and cyanuric acid were added as internal standards ($^{13}C_3$,$^{15}N_3$-melamine/cyanuric acid; Cambridge Isotope Laboratories, Andover, MA, USA) to attain a 20 μL concentration in the combined aqueous layer. The limit of quantitation of both analyses was 5 ppb, and linearity was up to 10 000 ppb. Between-batch precision coefficients of variation for quality control samples (10, 100, 400, and 1000 mg/L) were <10%, and recoveries from spiked standards into the blank matrix were >99%. The detection limits were 0.005 ppm in both serum/biological fluids and in tissue homogenate.

General maternal effects on pregnant rats, and adverse effects on foetuses and infants were monitored. In part I, bioavailability of melamine and cyanuric acid in both maternal and foetal/infant samples were measured by analytical methods. The pharmacokinetic model and parameters of melamine and cyanuric acid during pregnancy and in foetuses and infants were determined. In part II, the effective foetal and infant toxicity doses of melamine and cyanuric acid were derived. In part III, adverse maternal and foetal outcomes were recorded to determine the developmental toxicity potential of melamine and cyanuric acid.

Results

Maternal and foetal/infant pharmacokinetics

During pregnancy, single dose maternal exposure to melamine and cyanuric acid at about 0.2 and 20 mg/kg had no adverse maternal or foetal outcomes. Pharmacokinetics of melamine and cyanuric acid in amniotic fluid and the whole foetus followed the one-compartment model, but in maternal serum and breast milk followed the non-compartmental model. The pharmacokinetic profiles of melamine and cyanuric acid were similar. Cmax, AUC, T½, and MRTinf in maternal serum were significantly decreased in early gestation and significantly increased in late gestation and after delivery. However, the parameters were reversed in breast milk, amniotic fluid, and whole foetus in late gestation. Tmax in amniotic fluid, breast milk, and the whole foetus were longer than that in maternal serum. Cmax in maternal serum was the highest among the samples. Cmax in breast milk, the whole foetus, and amniotic fluid were lower than in maternal serum. AUC in maternal serum was the largest among the samples. The longest T½ was in breast milk, followed by amniotic fluid, the whole foetus, and maternal serum.

In infant rats, single dose maternal exposure to melamine and cyanuric acid at about 0.2 and 20 mg/kg had no adverse postnatal outcomes. The pharmacokinetics of melamine in infant serum and organs followed the non-compartmental model. In all infant samples, Tmax, T, and Vz/F were significantly decreased, but Cmax and AUC were significantly increased in later postnatal periods. Tmax was greater in infant kidneys than in serum; Cmax and AUC in the infant kidneys were much lower than in serum; and T½ in serum was shorter than in the kidney. The pharmacokinetics of melamine and cyanuric acid in foetal organs followed the one-compartment model, but infant organs followed the non-compartment model. Highest bioavailability was identified in foetal kidneys at late gestation and in infant kidneys at the early postnatal period.

Foetal and infant toxicity

Foetal LD_{100} was determined as 2500 mg/kg and infant IC_{100} as 320 mg/kg. Adverse outcomes after exposure to melamine at higher concentrations were more severe and common in early gestation than exposure to cyanuric acid in late gestation. Significantly more adverse maternal and foetal outcomes were encountered in early gestation, including acute toxicity, miscarriage, growth restriction, abnormal placentation, foetal resorption, but no congenital malformation was identified. Adverse neonatal outcomes secondary to higher concentrations of melamine were more severe and common at the pre-weaning period than following cyanuric acid exposure in the later postnatal period. Adverse maternal and foetal outcomes were significantly more in early gestation, including acute toxicity, hypoglycaemia, abnormal liver/renal function, proteinuria, haematuria and renal stones/crystals. The foetal LD_{50} was estimated as 300 mg/kg, whereas infant IC_{50} as <80 mg/kg and the tolerable daily intake as <0.02 mg/kg.

Reproductive toxicity

Maternal exposure of melamine and cyanuric acid resulted in no congenital malformation but significantly decreased maternal weight gain and litter size as well as increased

pregnancy loss and perinatal death. The adverse pregnancy and perinatal outcomes after exposure to melamine at higher concentrations were more severe and common than exposure to cyanuric acid. No abnormal renal function or renal stone/crystals were identified in maternal and foetal kidneys at concentrations up to 50 mg/kg.

Discussion

High bioavailability of melamine can pass through the placenta and enter foetuses and amniotic fluid as well as mammary glands and breast milk. Melamine is a small and highly polar compound. In breast milk and foetuses, it has a longer Tmax owing to delayed tissue distribution and compartment effects in maternal rats and the exocrine process of mammary glands. Transfer of melamine through the placenta to the foetal circulation and tissues may contribute to the delay in Tmax. Melamine then circulates in the foetus and is distributed in various tissues and excreted by the kidney into the amniotic fluid leading to further delays. As a result, in amniotic fluid the Tmax of melamine was the longest.

Melamine has a half-life in plasma of about 3 hours. It is then excreted in urine as the original compound. In neonate kidney that has just developed, the half-life is longer. Comparing the λz, the ability to remove melamine from maternal serum during pregnancy and the neonatal serum was similar. This suggests that accumulation of melamine may occur in the neonatal kidney, where water is reabsorbed and urine is concentrated before excretion.

Although melamine is quickly removed by urine, sub-chronic and chronic administration of melamine in adults could increase the incidence of ulceration of the bladder epithelium, inflammation, and epithelial hyperplasia of the urinary bladder, as well as the cancer rate of the urinary bladder and ureter.[7] Although there is no evidence of any teratogenesis due to melamine, triethylene melamine exposure has been shown to cause foetal death prior to or around the time of implantation.[8] Our reproductive toxicity study in the embryo and foetal development confirmed that there were adverse pregnancy and perinatal outcomes, but no definite teratogenic effects.

The US Food and Drug Administration published an Interim Safety and Risk Assessment of Melamine and its Analogues in Food for Humans in 2008. The human tolerable daily intake of melamine is suggested to be 0.63 mg/kg/day (ppm/day), whereas the tolerable daily intake recommended by the European Food Safety Authority is 0.5 mg/kg/day. The World Health Organization adopted the tolerable daily intake to be 0.2 mg/kg/day, which is applicable to both adults and infants as an estimated maximum amount of daily melamine exposure over a lifetime. In the present study, foetal LD_{50} for acute cardiac toxicity in utero was about 300 mg/kg, whereas infant IC_{50} for chronic renal toxicity was <80 mg/kg and its tolerable daily intake was <0.02 mg/kg.

In conclusion, maternal melamine and cyanuric acid could pass through the placenta and enter foetuses and mammary glands. It could also be eliminated through the placenta of the foetuses and the kidneys of the neonates. Melamine and cyanuric acid accumulated mainly in foetal and infant kidneys during late gestation and early postnatal periods. Neither melamine nor cyanuric acid was teratogenic, but reproductive toxicity during pregnancy was confirmed. This study provided information on the potential short-, medium- and long-term developmental toxicity associated with foetal and infant exposure to melamine and cyanuric acid, as well as toxicity reference data to regulate the human products to fulfil safety for pregnant/lactating women and infants.

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