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Citation	Hong Kong Medical Journal, 2013, v. 19 n. Suppl 8, p. 34-38
Issued Date	2013
URL	http://hdl.handle.net/10722/194728
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Impact of melamine-tainted milk on foetal kidneys and disease development later in life

背景：多種食品受三聚氰胺及其相關的物質的污染，政府有需要確保消費者的食物安全。
目標：評估胎兒接觸三聚氰胺和它的降解產物三聚氰酸的可能性，及接觸後對胎兒可能造成的後果。
方法：四個部分的目標如下：（1）使用細胞培養模型來研究三聚氰胺和三聚氰酸的交互細胞毒性；（2）檢測導致腎臟炎症和體內形成腎結石的能力；（3）利用人類胎盤灌注模型來評估母親與胎兒之間經胎盤的傳遞；和（4）透過動物模型闡明接觸三聚氰胺和三聚氰酸後，胎兒在子宮內及其早期生命階的短期與長期的涵義。
結果：三聚氰胺和三聚氰酸之間沒有出現任何加成效應或協同的交互作用。三聚氰胺能穿透胎盤到達胎兒，並在胎兒體內積聚，而三聚氰酸則沒有出現這種情況。在接受有三聚氰胺和三聚氰酸的老鼠胚胎內，並未發現三聚氰胺晶體或可觀察到的的毒性。
結論：三聚氰胺能穿過人類胎盤。
涵義：孕婦接觸三聚氰胺和三聚氰酸後，這些物質能傳遞至成長中的胎兒。由於污染物對人類具毒性，接觸後對胎兒可能會構成重大的風險，因此三聚氰胺的毒性機制在胎盤中造成的毒性影響需要進一步研究。

Key Messages

1. Melamine has very low cytotoxicity on kidney cells of a susceptible species (canine), and the presence of its main degradation product cyanuric acid did not influence its cytotoxicity. This indicates that the toxicity of melamine is mainly due to the formation of kidney stones rather than a direct toxic effect on kidney cells.
2. Ingestion of melamine alone failed to induce kidney stones even under conditions of restricted drinking water access. In mice administrated with melamine together with cyanuric acid, no renal stones were formed when the supply of drinking water was unrestricted. However, when drinking water was limited, stone formation was observed.
3. Melamine was detected in the foetal circulation of perfused human placenta and easily transferred through it. The presence of cyanuric acid did not influence the transfer of melamine from the maternal to the foetal side.
4. Administration of melamine and cyanuric acid to pregnant mice did not cause any noticeable developmental or reproductive abnormalities.
5. No melamine crystals were detected in kidneys of the embryos or breastfed pubs, despite the mothers having melamine crystals in their kidneys and renal failure.

Hong Kong Med J 2013;19(Suppl 8):S34-8

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Introduction

Consumption of melamine-tainted milk products has affected tens of thousands of Chinese children. Up to 27 November 2008, 294 000 children were reported to show urinary system abnormalities, of whom 51 900 were hospitalised. It is uncertain whether such children will incur other complications such as tumourigenesis or growth retardation in the future.

Due to immaturity and quickly developing organs, foetuses may be highly susceptible to the effects of environmental toxins.[1-4] There is association between growth and health of the foetus and infant and the risk of several diseases later in life. Transplacental transfer of toxic compounds via the human placenta is important for foetal risk assessment. Studies on human placenta are crucial because of functional differences in placental anatomy and physiology between different species.

In China, infant milk formula contaminated with melamine and cyanuric acid (CA) was taken by the children. These compounds have been found in food products (milk and other dairy products, eggs, chicken) that were consumed in high amounts. There is potential toxicity from consuming melamine in combination with its degradation products. This study aimed to investigate the impact of possible synergistic effects between melamine and CA and the potential of transplacental passage of melamine and its consequences on foetuses and disease development later in life.

Methods

This study was conducted from April 2009 to December 2011 and was divided into four parts to study: (1) interactive cytotoxicity of melamine and CA using Madin-Darby canine kidney cells, (2) induction of renal inflammation and kidney stones in vivo in mice, (3) the maternal foetal transplacental passage using an

ex vivo human placenta perfusion model, and (4) short and long-term implications of exposure to melamine and CA in utero and during early stages of life in a mice model.

Results

Study I: interactive toxicity of melamine and its degradation product in a cell culture model

When melamine was mixed with CA in different ratios of 1:1, 10:1, 100:1, and 1000:1, and then incubated with Madin-Darby canine kidney cells for 48 hours, there was a very weak cytotoxic effect (as measured by the cell viability); less than 20% of the cells were adversely affected. The data obtained from the cytotoxicity assays of melamine and CA was compared with the effect of melamine and CA alone (Fig 1).

Study II: induction of renal inflammation and kidney stones in vivo in mice

In mice, ingestion of melamine alone failed to induce kidney

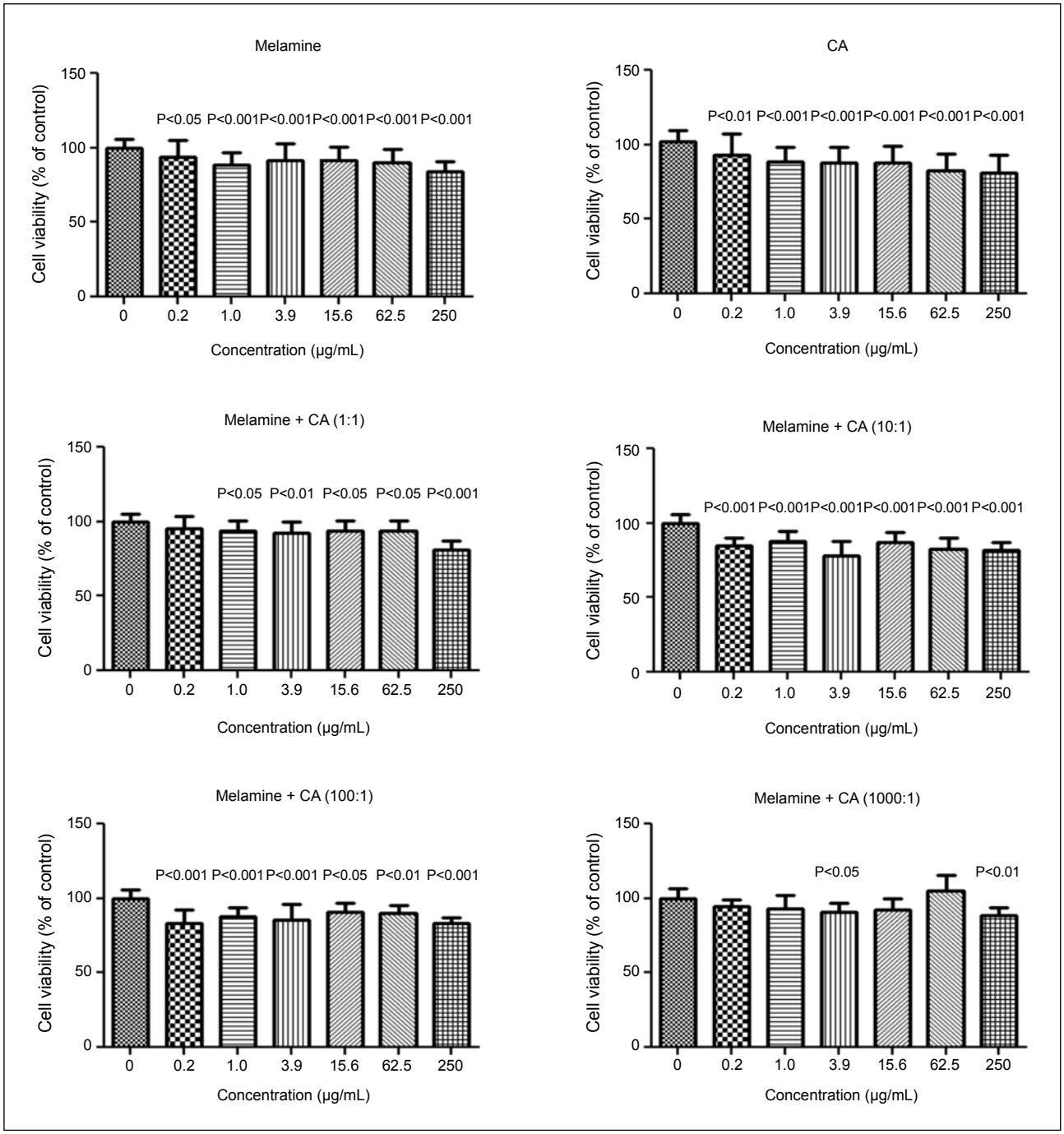

Fig 1. Individual and combined cytotoxicity of melamine and cyanuric acid (CA) after 48 hours of exposure. At least two independent experiments with six data points were run for each test chemical

stones even under conditions of restricted drinking water. When melamine was administrated with cyanuric acid for 3 days, no renal stones were formed when drinking water was unrestricted, but when drinking water was limited, stone formation accompanied by high levels of serum urea and creatinine were observed (Fig 2).

Study III: maternal foetal transplacental passage using an ex vivo human placenta model

When placentas were perfused with 10 μM melamine, approximately 0.15% of that concentration was detected in the foetal circulation after 5 minutes. The amount of melamine increased in the foetal circulation and decreased in the maternal circulation over time and at the end of the 4-hours perfusions, a mean of 62% and 39% of the original melamine concentration were detected in the maternal and foetal circulation, respectively.

As a rat study indicated that melamine transfer through placenta is dose dependent,[5] additional perfusion experiments with 1 mM melamine were carried out to investigate whether there was a difference in the transfer based on concentrations. Transfer of 1 mM melamine did not differ significantly.

Because melamine contaminated products may contain CA, perfusions were carried out with a mixture of 10 μM melamine with 10 nM of CA, to investigate whether CA affected the transfer of melamine. Transfer of melamine in these perfusions was similar, irrespective of the CA concentrations.

Study IV: short- and long-term implications of exposure to melamine

There were no significant changes in the number of mouse embryos in the control and melamine mothers. However, embryos in the latter mothers were slightly smaller in size. Morphological and histological examination of the embryos revealed no obvious bleeding or other abnormalities, and the blood vessels were well developed (Fig 3).

Discussion

Although the pathophysiology of urinary stones secondary to exposure to both melamine and CA has been reported, their combined action on kidney cells remains unknown. Study I screened for interactive toxicity of melamine and CA using a cell culture model. No additive or synergistic interactions were noted when mixed in ratios of 1:1, 10:1, 100:1, and 1000:1. Melamine generally appeared to reduce the harmful acidic effects induced by CA.

The effects of melamine and CA uptake on kidney stone formation have been reported in fish, cats, dogs, and pigs, but not in mice. Results from study II and others indicated that melamine administration alone without CA cannot induce the renal stone formation in experimental animals. When melamine and CA were administered together for 3 days, melamine crystals were found in the kidneys together with acute renal failure. However, this only occurred when access to drinking water was limited. Under conditions of unrestricted drinking water, ad libitum crystals were found very occasionally, and no dilated tubules were observed in

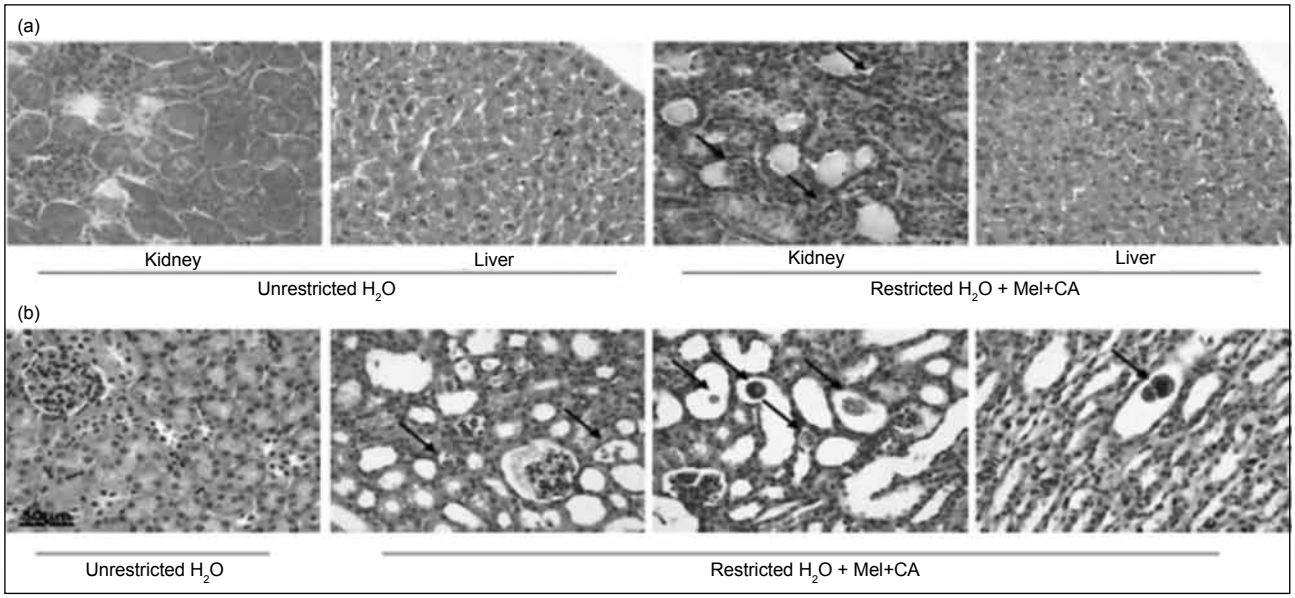

Fig 2. Histology analysis of melamine stone formation in the kidney in frozen section stained with H&E: (a) frozen sections of the kidney and liver from mice fed with melamine and cyanuric acid (CA) under restricted access to drinking water (Restricted H_2O + melamine + CA) were compared to water delivered ad libitum (Unrestricted H_2O). (b) Paraffin sections of mouse kidneys under restricted access to drinking water were compared to unrestricted drinking water controls. From left to right, cortical regions of Unrestricted H_2O group and the glomeruli, tubules and medullary regions of the Restricted H_2O + melamine + CA group. Scale bar represents 50 μm. Melamine stones are indicated with arrows

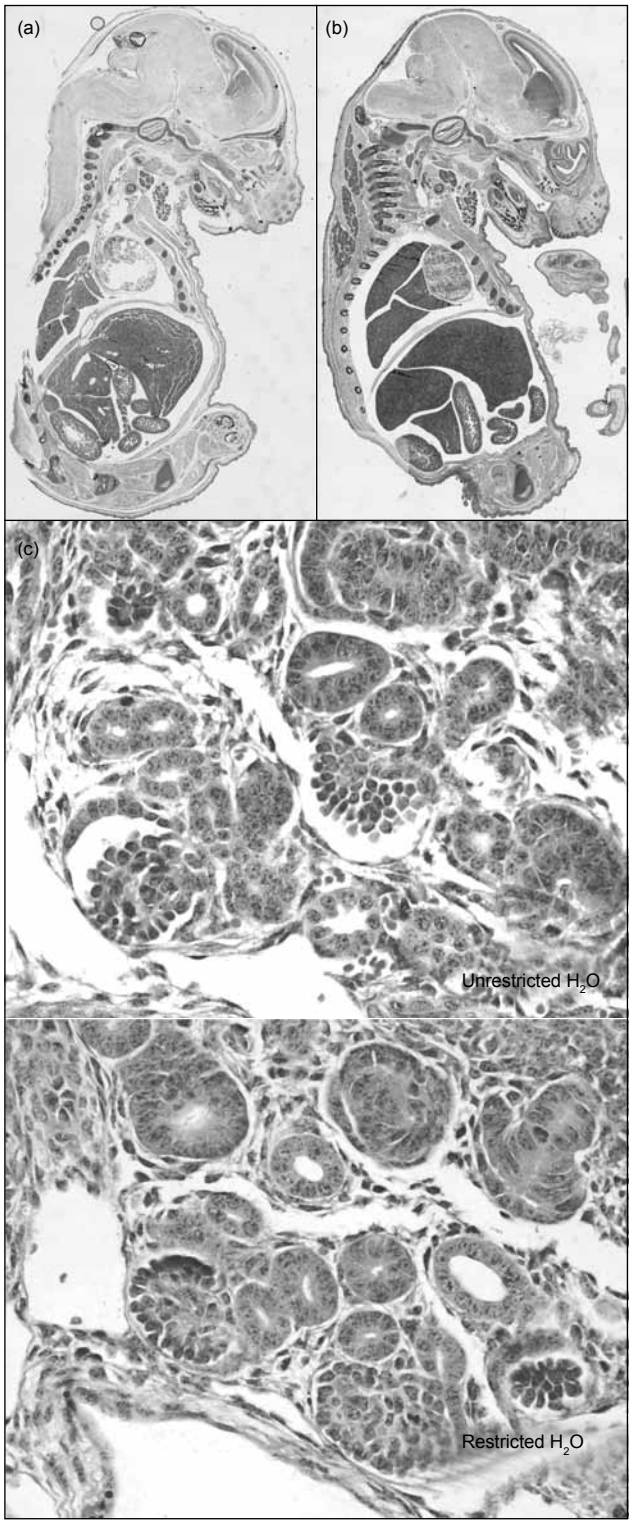

Fig 3. Cross-sections of mouse embryos stained with H&E: (a) pregnant mouse control and (b) pregnant mouse administrated with melamine and cyanuric acid (400 and 40 mg/kg/day for 3 days). (c) Cross-sections of embryo kidneys from pregnant mouse administrated with melamine and cyanuric acid

tissue sections and the mice had no symptoms of kidney failure. The half life of melamine in the blood is about 2.7 hours and it is cleared mainly through renal system. After restricting drinking water, melamine and CA were retained longer with higher concentrations in the kidney, enabling the two chemicals to interact and form crystals. In addition, the reduction of kidney function by crystals further limited the water exchange and the kidney failure occurred rapidly. Our results partially explained how melamine stones cause acute kidney failure in patients and animal studies.

During pregnancy, the placenta develops and its thickness and cell layers decrease from >50 μm at the 2nd month to <5 μm at the 37th week of pregnancy. Due to the changes in placenta, transplacental transfer probably varies during the course of pregnancy and because it is thinnest at term, drug transport may also be highest.[6] Study III is the first report on melamine transfer on human placental perfusion and provides the evidence of a fast transfer through the term human placenta. Melamine crossed the placental barrier quickly, as indicated by the presence of small concentrations (0.12 to 1.34%) in the foetal circulation 5 minutes after the addition to the maternal circulation; concentrations exceeded 34% after 4 hours of perfusion. One study on the transfer and accumulation of melamine in rat foetuses and placentas suggested that the transfer of melamine is dose dependent.[5] In our study, there was some indication of slightly quicker transfer with higher concentrations, but the difference was not significant. The kinetics of melamine clearly differed from those of antipyrine, which diffuses passively through the placenta. Transfer of melamine was significantly slower, implicating the involvement of other contributing factors such as the presence of placental efflux transporters.

Results from study IV using mouse and alternative water supply to investigate the effect of melamine and CA on embryo development suggested that stones might only be formed in a functioning kidney. As embryonic kidney has no function, hence no stone was detected in embryos from pregnant mice exposed to melamine and CA.

Acknowledgements

This study was supported as part of the Studies Related to Melamine Incident commissioned by the Food and Health Bureau, Hong Kong SAR Government (MI-BS-16). We thank Pro Kirsi Vahakangs, Prof Seppo Auriola from the School of Medicine, University of Eastern Finland, Prof Seppo Heinonen and staff of the maternity ward in Kuopio University hospital for the placental perfusion studies.

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