Original Article

Melamine Nephrotoxicity is Mediated by Hyperuricemia*

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Abstract

Objective We tested whether melamine nephrotoxicity was exacerbated by urate (a typical component of renal stones in humans) in rats with hyperuricemiainduced by the uricase inhibitor, potassium oxonate (Oxo).

Methods Rats were exposed to melamine or Oxo alone or combinations of melamine (200-400 mg/kg) and Oxo (200-600 mg/kg) for 3 consecutive days. Kidney injury was evaluated by renal biochemical functions, histomorphology, and lipid peroxidation. Kidney crystals were analyzed for their composition.

Results Nephrotoxicity was minimal in animals administered melamine or Oxo alone, but it was demonstrable in animals administered at least 800 mg/kg of the two compounds combined. All rats in the 400+600 (melamine+Oxo) and 400+400 mg/kg groups and 4 out of 6 in the 200+600 mg/kg group died within 3 days; no rat died in the 200+400 or 200+200 mg/kg group. Dose-dependent renal damage resembling clinical findings in affected patients was observed in rats administered the two compounds. Crystal composition determination revealed the existence of melamine and uric acid in the affected kidneys, resembling human stones.

Conclusion Our findings suggest that uric acid plays a key role in melamine-related kidney injury in humans. Future studies should consider uric acid together with melamine when examining adverse effects in humans.

Key words: Melamine; Nephrotoxicity; Hyperuricemia; Urolithiasis

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INTRODUCTION

The melamine-adulteration event in China has received substantial public health attention, with approximately 300,000 affected children suffering from urinary tract abnormalities[1-3]. Although most of the affected children have recovered[1,4], concerns remain over the potential long-term adverse effects of melamine exposure, particularly considering kidney injury during childhood has been associated with chronic disease in later life[5-6]. It is unclear what longitudinal

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follow-up of these children is required, because of the absence of a proper animal model from which to extrapolate to potential human consequences.

It long had been believed that melamine was a chemical with limited toxicity and without renal toxicity even at very high doses [oral lethal dose (LD_{50}) approximately 3100-3300 mg/kg in rodents][7-8]. This notion remained largely unchallenged until the 2007 pet food scandal in which the toxicity of melamine was augmented drastically and was thought to be due to the coexistence of cyanuric acid (CYA), which is a less toxic analogue of melamine[9]. Thereafter, various melamine-CYA models were established to simulate the renal toxicity of melamine in humans[8-10]. However, the amount of CYA in contaminated infant formula milk products, unlike that in the contaminated pet food, was extremely low (approximately 0.1% of melamine)[8]. Although additional CYA can be produced by microbial transformation of melamine[10], the amount of CYA in the kidney stones of affected children was substantially lower than that in the kidney stones of animals[11]. This suggests that human melamine-related stones areformed primarily through other pathways.

Melamine and urate are two predominant components in human kidney stones that have been hypothesized to interact in the formation of kidney stones in patients[11]. This hypothesis has not been tested *in vivo*, although it seems plausible considering humans have higher uric acid levels, possibly due to the absence of the uricase enzyme that exists in animals[12]. In this study, we aimed to test whether melamine could cause human-like nephrotoxicity in rats with high uric acid levels that were induced by a nontoxic uricase inhibitor, i.e., potassium oxonate (Oxo). The results revealed that the nephrotoxicity of melamine was greatly exacerbated in hyperuricemic rats.

MATERIALS AND METHODS

Experimental Animals

Adult healthy male Wistar rats of clean grade weighing 200±20 g were purchased from Vital River (Beijing, China). The rats were housed under controlled temperature (20±2 °C) and humidity (55%±5%) in an environment of 12-h light/12-h dark cycle. Standard laboratory food and water were available ad libitum. The rats were acclimatized for 3 d before the experiment. The rats were allocated randomly to each group using random numbers created by Microsoft Excel (Microsoft Corp., Redmond, WA, USA). During the housing, the animals were monitored once daily for the health status. The hyperuricemic model and melamine toxicity studies were each conducted using 48 rats (n=6 in each group). All experimental protocols were approved by the Ethics Committee of Peking University Health Science Center (Permit Number: 00001052-11012), and in accordance with the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006). All efforts were made to minimize suffering.

Oxo-Induced Hyperuricemic Models

Two batches of rats were used to evaluate the effects of Oxo on uric acid levels as well as its renal toxicity. In one batch, 24 rats were divided randomly into 3 dosing groups and a control group. The rats in the dosing groups received daily intraperitoneal injection of Oxo (>97%; Sigma-Aldrich, St. Louis, MO, USA) for 3 consecutive days at doses of 200, 400, and 600 mg/kg, respectively. The rats in the control group received the carboxymethyl cellulose (CMC, 1%; Dingguo, Beijing, China) vehicle. Blood was collected from the orbital vein at 0, 1, 2, 3, 4, and 5 h after dosing on day 1. After dosing for 3 consecutive days, the kidneys were excised and fixed in formalin (10%) for paraffin sections. In the other batch, 24 rats were divided randomly into 4 groups (0, 200, 400, 600 mg/kg Oxo) for 4 consecutive days. Urine (24 h) was collected before dosing and on the first 3 dosing days. On dosing day 4, blood was collected from the orbital vein at 0, 1, 2, 3, 4, and 5 h after dosing.

Melamine-Urate Models

There were 48 rats randomized into 8 groups, including one control group (1% CMC), one Oxo alone group (600 mg/kg, intraperitoneal injection), one melamine alone (200 mg/kg, oral gavage, >99%; Sigma-Aldrich), and 5 combined dosing groups of melamine and Oxo (200+200, 200+400, 200+600, 400+400, and 400+600 mg/kg). In the combined dosing group, melamine was administrated immediately after Oxo injection. The dosing lasted for 3 consecutive days. Deaths were recorded during the 3 dosing days, and 24-h urine was collected. The survived rats were sacrificed and necropsied on day 4; one kidney was photographed, weighed, and fixed in formalin (10%) for paraffin sections, and another was freeze-dried for composition analysis.

Kidney Composition Analysis

To perform the electrospray ionization time-of-flight mass spectrometer analysis (ESI-TOF-MS; Xevo G2-S Qtof, Waters, Milford, MA, USA), freeze-dried kidneys were ground to powder and dissolved in a tube with 4 mL of acetonitrile and 2 mL of hexane. The tube was vortexed for 10 min and centrifuged at 6000 rpm for 10 min. The supernatant was removed, and the remains were analyzed by ESI-TOF-MS equipped with an electrospray source and lockspray, running in positive (ES+) and negative (ES−) modes.

Freeze-dried kidneys were ground to powder for the extraction of melamine and uric acid. Melamine was measured using high performance liquid chromatography (HPLC; Agilent 1200; Agilent Technologies, Santa Clara, CA, USA) coupled with mass spectrometry (MS/MS; Agilent 1200 6410; Agilent Technologies) with modified procedures[10]. Then, 100 mg powder was weighted into a 15 mL tube; 5 mL trichloroacetic acid (TCA, 3%), 2 mL acetonitrile (Fisher Scientific, Waltham, MA, USA), and 2 mL hexane (Fisher Scientific, Waltham, MA, USA) were added. The tube was vortexed for 30 s, sonicated for 10 min, and then centrifuged for 5 min at 8000 rpm. The supernatant was filtered by the hybrid cation-exchange solid-phase extraction column (Cleanert PCX-SPE; Agela Technologies, Wilmington, DE, USA) that had been conditioned by 3 mL methanol and 3 mL water. Melamine was eluted by 5 mL ammoniated methanol (ammonia water:methanol=1:19, v/v) and evaporated dry under nitrogen at 60 °C. The dried extract was reconstituted by 1 mL mixture of water: acetonitrile (1:19, v/v) for HPLC-MS/MS analysis. Melamine standard solutions (2, 5, 10, 25, 50, and 100 ng/mL) were prepared with the same mixture of water and acetonitrile; the r^2 of the standard curve was >0.999.

Uric acid was measured using HPLC (Agilent 1200; Agilent Technologies) coupled with the diode array detector (DAD; Agilent 1290 Infinity; Agilent Technologies) at 280 nm. Then, 20 mg powder was weighted into a 15 mL tube; then, 10 mL water: acetonitrile (1:2, v/v) and 3 mL hexane were added. The tube was vortexed for 10 min and centrifuged at 6000 rpm for 10 min. The supernatant was removed, and the sediment was diluted 5 times by water: acetonitrile (1:19, v/v) before quantitative analysis. Uric acid standard solutions (2, 5, 10, 25, 50, and 100 ng/mL) were prepared with the same water: acetonitrile (1:19, v/v)mixture; the $r^2$of the standard curve was >0.999.

Histological Examination

The formalin-fixed kidneys as aforementioned were embedded in paraffin after ethanol dehydration, and then were cut into 5-μm sections for H&E staining and immunohistochemistry following standard protocols[10,13].

Malondialdehyde(MDA) Measurement

MDA in urine was measured by using the thiobarbituric acid reactive substances (TBARS) Assay Kit (Cell Biolabs, San Diego, CA, USA) following the assay instructions. The r^2 of the standard curve is >0.999.

Biochemical Examination

Serum uric acid, serum blood urea nitrogen (BUN), serum creatinine(SCr), urine urea nitrogen (UUN), urine creatinine(UCr), and urine osmolality (Uosm) were measured using an Olympus automated biochemistry analyzer (Olympus AU400; Shinjuku Monolith, Tokyo, Japan).

Statistical Analysis

The levels of MDA and the changes in the levels of serum uric acid and renal function indicators (BUN, SCr, uric acid, UUN, UCr, and Uosm) from baseline (before dosing) were presented as $x \pm sx$. Between-group comparisons were performed using the one way analysis of variance followed by the Dunnett's test for multiple comparisons or using Kruskal-Wallis analysis of variance test followed by theNemenyi test for multiple comparisons, as appropriate. SPSS 20.0 was used for data analysis. A two-sided P value less than 0.05 was considered significant.

RESULTS

Hyperuricemia Induced By Oxo

We used various doses of Oxo alone for 4 consecutive days to elevate uric acid concentrations in rats. On day 1 of dosing, the serum uric acid level was elevated rapidly by Oxo injection, and then reduced sharply within 5 h in a dose-dependent manner (Figure 1). Maximal hyperuricemia occurred at 1, 1-2, and 2 h for the 200, 400, and 600 mg/kg groups, respectively. Compared to controls, significant elevations were observed at 4 of the 5 time-points (except 4 h) for the 600 mg/kg group and at the first 3 time-points for the 400 mg/kg (all P<0.05). Similar results also were observed on day 4 of Oxo injection (Figure 1, Table S1).

The Renal Effects of Oxo

The levels of UUCr, UUN, Uosm, BUN, and SCr were measured to reflect renal functions after 3 dosing days. The renal effects of Oxo alone were minimal. Except for a decreased Uosm in all Oxo alone dosing groups, none of UCr, UUN, SCr, and BUN in the 200 and 400 mg/kg groups was significantly different from that in the corresponding control groups (Figure 2). Doses of 600 mg/kg Oxo caused higher SCr and BUN than in the control group after 4 consecutive days (P<0.05).

Morphological and histological changes also were examined. Compared to the control group, no obvious changes were observed in HE-stained pathological sections (Figure 3a3, 3b3) in rats administered 600 mg/kg Oxo alone for 3 consecutive dosing days. The kidney in the Oxo alone group was slightly larger than that in the control group; however, no brownish color was observed in either group (Figure 3a1, 3b1, 3a2, and 3b2).

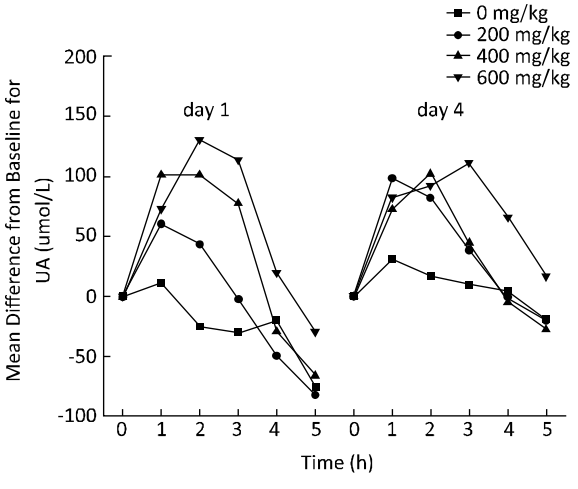


Figure 1. Hyperuricemia induced by Oxo. Changes in the levels of serum uric acid for each time point in comparison to baseline (before dosing) were presented after rats were injected intraperitoneally with potassium oxonate (Oxo). On day 1, significant differences were observed at all 4 time-points (except 4h) for the 600 mg/kg group and at the first 3 time-points for the 400 mg/kg group as compared to the corresponding levels in the control group (all P<0.05). On day 4, a significant difference was observed at 3 h for the 600 mg/kg group as compared to the control group (P<0.05).

Lipid Peroxidation after Oxo Injection

MDA in urine was measured as an index of lipid peroxidation. The levels were similar among the control and Oxo alone dosing groups after 3 consecutive dosing days (Figure 4). However, the within-group variations were more pronounced in the Oxo alone groups than in the control group.

The Renal Effects of Melamine Plus Oxo

Before the study of *in vivo* crystal detection, a saturated solution of melamine and Oxo (pH of both were adjusted at 5.5 with HCl and NaOH) was mixed and incubated at room temperature overnight to test if the crystals would be produced. We found that the mixed solution did not produce any visible crystals on the next day.

Melamine plus Oxo at higher doses was fatal (Table 1). All 12 rats exposed to melamine plus Oxo at 400+600 and 400+400 mg/kg died over 3 dosing days, while 4 out of 6 rats died in the 200+600 mg/kg melamine plus Oxo group. No rats died in the dosing groups of 200+200 or 200+400 mg/kg or in the control group.

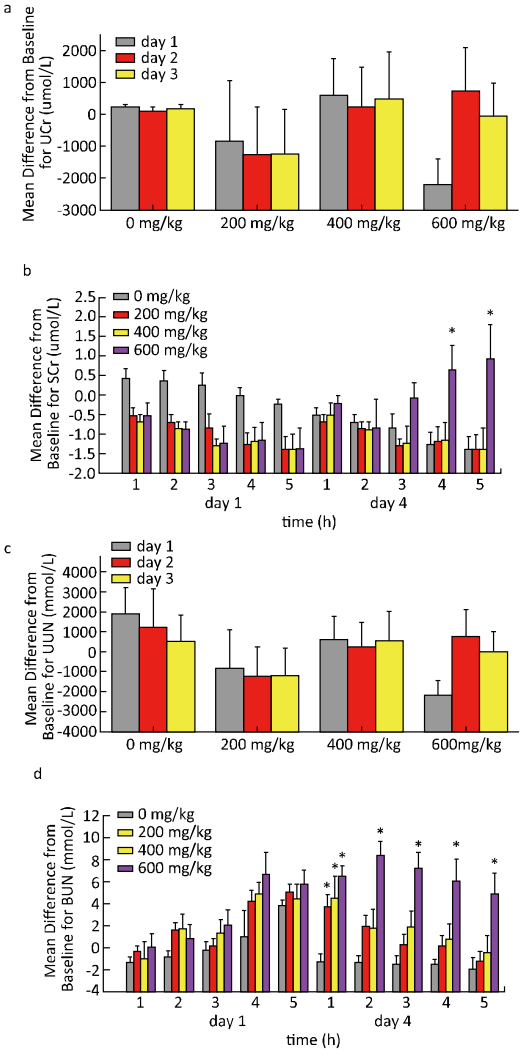
The effects of melamine plus Oxo on histological changes were assessed after 3 consecutive dosing days. Morphological changes were observed in surviving rats administered melamine and Oxo (200+200, 200+400, and 200+600 mg/kg) but not in the controls, including brownish color, enlarged size, and uneven surfaces with needle-like brown-colored spots (Figure 3 c1-e1); in longitudinal sections, brown-colored strings were observed (Figure 3 c2-e2). The spots and strings were expanded gradually to the whole kidneys with increasing doses of Oxo. Under the microscope, tubule-obstructed pathological changes were observed, including dilated renal tubules with visible hyperemia, interstitial vascular dilation, and hydropic degeneration in proximal tubule epithelial cells (Figure 3 c3-e3). Brownish needle-like crystals radially aggregated in tubular lumina were observed in the 200+600 mg/kg group (Figure 3 e3).

Lipid Peroxidation after Administering Melamine Plus Oxo

Urine MDA levels were measured after 3 consecutive dosing days. The level in the melamine plus Oxo group (200+200 mg/kg) was higher than in the control group; however, the differences were not significant (P>0.05; Figure 4).

Melamine and Uric Acid In Affected Kidneys Administering Melamine Plus Oxo

In the ESI-TOF-MS spectrometry analysis of affected kidneys of melamine-urate rats, we observed two typical peaks at *m/z*=126 and 168, indicating the existence of melamine and uric acid (Figure 5). Melamine and uric acid concentrations in the affected kidneys of survived rats were measured further using HPLC-MS/MS and HPLC-DAD, respectively. The mean melamine concentration varied slightly, yet in a dose-dependent manner, across dosing groups (1.36-1.68 mg/g dried kidney); the concentrations were substantially higher than those in the control group. Uric acid concentrations increased from 5.35 to 12.58 mg/g with increasing Oxo doses; uric acid was not detected in the control group (Table 1).



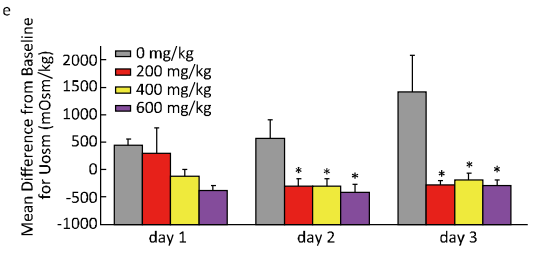


Figure 2. Effect of Oxo injection on renal functions. (a) The urine creatinine (UCr) levels remained constant over the 3 dosing days in the control and 200 and 400 mg/kg groups, whereas in the 600 mg/kg group, there was a nonsignificant elevation in UCr levels on the days 2 and 3. Compared to the control rats, the within-group variations in UCr levels were more pronounced in dosing rats. (b) On dosing day 1, SCr showed a tendency to decrease from 1 to 5 h after Oxo injection on day 1; the SCr level in the control group tended to be higher than that in the Oxo dosing groups; however, the differences were not significant. On day 4, a similar decreasing tendency was observed in the controland 200 and 400 mg/kg groups, whereas the SCr levels showed an increasing trend in the 600 mg/kg group, with significantly higher levels at 4 and 5 h compared to the controls (P<0.05). (c) The UUN in the control group showed a slightly decreasing trend in the 3 dosing days, and the levels in the 200 and 400 mg/kg groups remained almost unchanged. The levels in the 600 mg/kg group tended to be higher on days 2 and 3 than on day 1, yet the differences were nonsignificant. (d) On dosing day 1, serum BUN showed a tendency to increase from 1 to 5 h after Oxo injection on day 1; the levels in control group tended to be lower than that in the Oxo dosing groups; however, the differences were not significant. On dosing day 4, the BUN levels in the 3 dosing groups were significantly higher than in the control group at 1 h after Oxo injection. (e) The Uosm in the 3 dosing groups was significantly lower than that in the control group on dosing days 2 and 3 (P<0.05). On day 1, there also was a tendency of decreased Uosm in the 400 and 600 mg/kg groups; n=5 per group. $^{*}P$<0.05 compared to the control group.

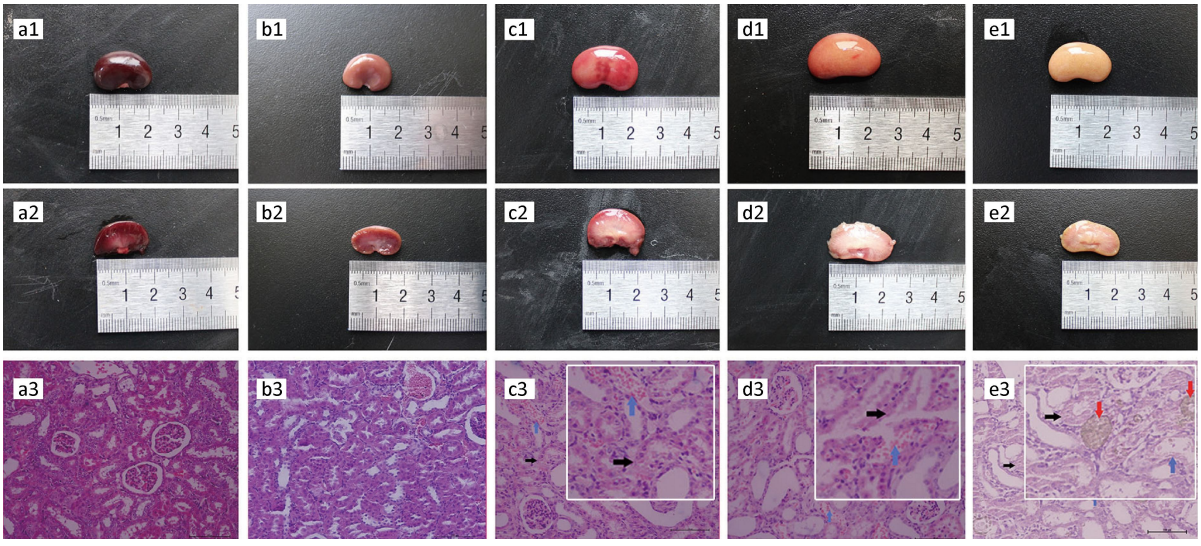


Figure 3. Gross appearance and histological examination of the kidney after dosing for 3 consecutive days (×200). (a1-e1) Gross appearances of kidneys for the control group (1% CMC), 600 mg/kg Oxo alone group, and the 3 combined groups of melamine and Oxo (200+200, 200+400, and 200+600 mg/kg). (a2-e2) Longitudinal sections of kidneys for the corresponding 5 groups. (a3-e3) Representative photographs of histological examination for the corresponding 5 groups. Hyperemia (*blue arrow*) and hydropic degeneration (*dark arrow*) of proximal tubule epithelial cells were observed. Golden-brown colored crystals (*red arrow*) also were observed in renal tubules in the 200+600 mg/kg group (e3).

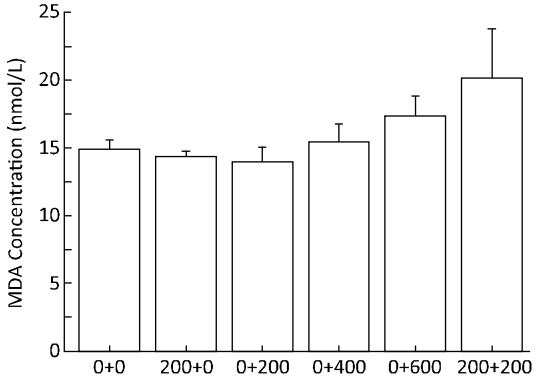


Figure 4. Lipid peroxidation after 3 dosing days.Urine MDA level for control rats and for rats administered melamine alone, Oxo alone, or melamine plus Oxo. *n*=5 for each group.

DISCUSSION

In the present study, we found that Oxo significantly elevated uric acid concentrations with mild renal toxicity. The combination of melamine and Oxo caused severe renal toxicity that was lethal in animals receiving a high combined dosage of Oxo and melamine. The two agents together provoked a pattern of renal injury that resembled clinical findings described in patients. Moreover, the damage occurred in a dose-dependent manner. We also found that relatively lower doses of melamine and Oxo caused renal damages but were nonlethal, suggesting that such models could be used for further investigation into the effects of melamine-induced injuries on later health outcomes.

Most mammals have the uricase enzyme that can degrade uric acid to allantoin[12]. Oxo, as anuricase inhibitor, has been used to generate hyperuricemic models[14-16]; however, few studies have quantified the uric acid elevation patterns shortly after Oxo injection, essential to establish the present melamine-urate model. In our study, we found that 200 to 600 mg/kg Oxo injection could induce hyperuricemia within 1 h that lasted at least 3 h, close to the half-life of melamine[17]. We also observed that the renal toxicity of Oxo was quite mild, even at higher doses, characterized by abnormal BUN and SCr, and reduced Uosm with no elevation in UUN and UCr or histological changes. These features suggest that this Oxo-induced hyperuricemia model is feasible for investigating the renal toxicity of melamine under high uric acid status.

Table 1. Uricacid and Melamine Content in Rat Kidney 3 Days after Administering Melamine Plus Oxo

Melamine (mg/kg)	Oxo (mg/kg)	Number of Survivals	Gross Morphological Changes Scores*	Histological Changes†	Uric Acid (mg/g Dried Kidney)[a]	Melamine (mg/g Dried Kidney)[a]
400	600	0/6	+++	+++	*NA*[b]	*NA*
400	400	0/6	+++	+++	*NA*	*NA*
200	600	2/6	+++	+++	12.58±0.57	1.68±0.04
200	400	6/6	++	+	8.04±0.86	1.58±0.19
200	200	6/6	+	+	5.35±0.05	1.36±0.00
0	0	6/6	-	-	0	0.03±0.00

Note. *Gross morphological changes include changes in color and size. (+++ was defined as golden-brown color of the whole kidney surface and longitudinal section; ++ was defined as brown color of the whole kidney, and brown color was confined within only medulla from longitudinal sections; + was defined as scarce brown color of the kidney surface, brown color was confined within only longitudinal section). †Histological changes includes hyperemia, interstitial vascular dilation, and hydropic degeneration (+++ was defined as obvious tubular dilation, hyperemia, and interstitial vascular dilation; ++ was defined as medium tubular dilation, hyperemia, and interstitial vascular dilation; + was defined as scare tubular dilation, hyperemia, and interstitial vascular dilation). [a]The uric acid and melamine contents were measured only in the survived rats. The uric acid and melamine were measured for two kidney samples per group. [b]*NA* not analyzed.

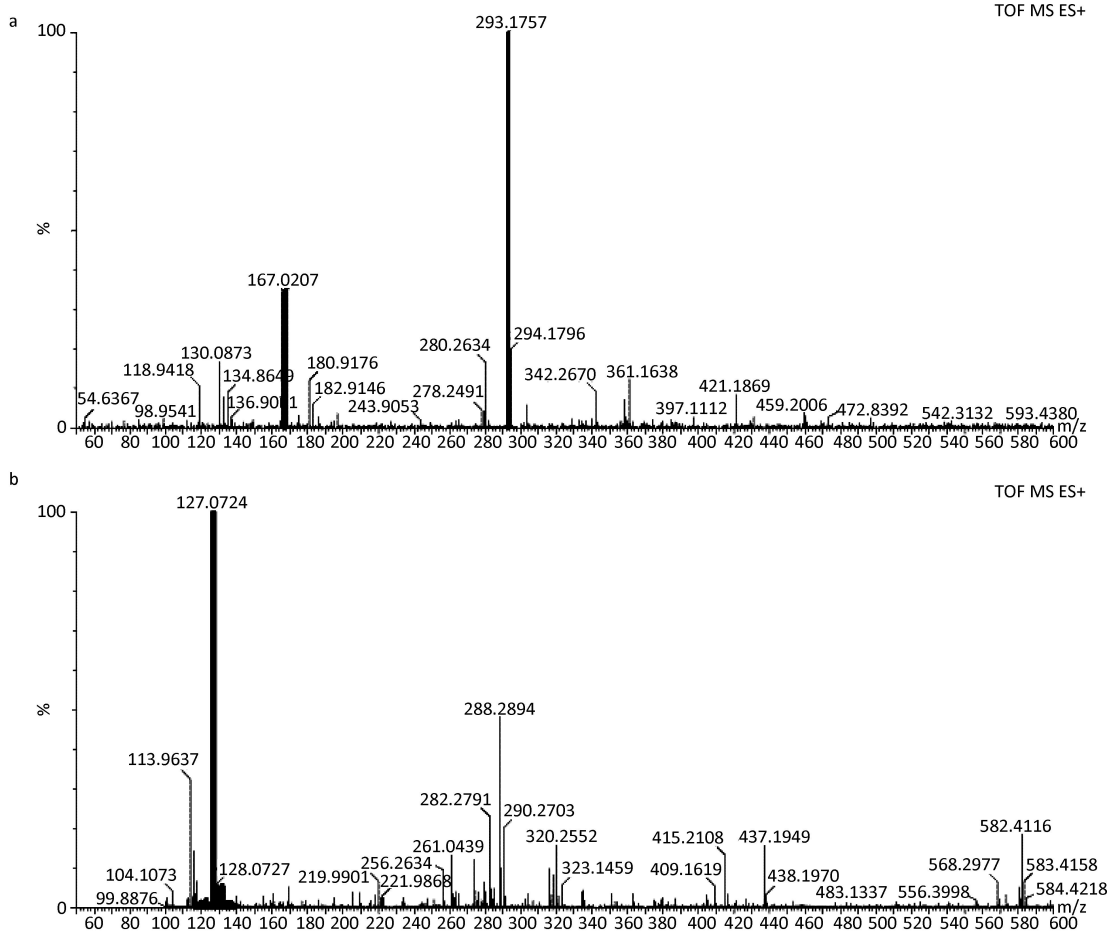


Figure 5. ESI-TOF-MS spectrometry of the kidney of rats administered melamine and Oxo (200+600 mg/kg). (a) Typical TOF mass spectra running in negative mode. (b) Typical TOF mass spectra running in positive mode. There were two typical peaks at *m/z*=126 and 168, likely representing melamine and uric acid, respectively.

Our findings of mild renal effects of hyperuricemia alone are comparable with the extent of kidney injury observed in mice with liver specific knockout of the urate transporter, Glut9 [18].

Melamine when provided alone is nontoxic[11]. In our study, when provided to hyperuricemic rats, the renal toxicity of melamine was augmented drastically. The dose-dependent renal damages, from gross appearance to histological changes, were similar to human findings[2,19]. The concentrations of melamine and uric acid in the affected kidneys of dosing rats were much higher than those in control rats, further indicating an interaction between the two compounds and a strong similarity to the pattern of human kidney damage[11].

Unlike our study focusing on uric acid, previous animal experiments exclusively focused on the role of CYA in augmenting renal toxicity of melamine[7,9]. Upon confirming the augmented effect of melamine by CYA, researchers have attempted to elaborate the role of CYA in the human tragedy, although the CYA in contaminated dairy products was extremely low. In a recent study, Zheng et al.[10] demonstrated that CYA, as a co-crystallizing chemical derivative of melamine, can be produced from melamine under the metabolic effect of gut microbiota, which provides new insights into the pathogenesis of the nephrotoxicity of melamine in humans. Could the enhanced renal toxicity be explained by the auto-transformed CYA? Zheng et al.[10] observed no obvious changes in gross kidney appearances after the daily administration of 600 mg/kg melamine for over 2 weeks. In chronic toxicity experiments, much higher doses of melamine (2250 mg/kg) administered for over 2 years remained nonlethal to rats[20]. In contrast, in the China melamine- contamination event, where 6 children died and approximately 50,000 were hospitalized due to renal injuries, the average amount of melamine consumed was <40 mg/kg per day[1], suggesting that the nephrotoxicity of melamine could not be explained satisfactorily by microbial transformation of melamine to CYA.

Oxo is structurally similar to CYA, and, therefore, might precipitate with melamine to induce nephrotoxicity in rats. This pathway, however, seemingly was not supported by the fact that when saturated solutions of melamine and Oxo were mixed and incubated at room temperature overnight, no crystals were identified visually. Therefore, the nephrotoxicity of melamine plus Oxo could be ascribed largely to the melamine-urate pathway.

In the present study, relatively lower doses of melamine, when provided to hyperuricemic rats for only 3 d, demonstrated severe renal toxicity. This hyperuricemia-based rat model, when considered in the context that the main components of human kidney stones are melamine and urate, may replicate more closely the toxicity of melamine in humans. We suggest that the melamine-induced renal toxicity in hyperuricemic rats results from the physical obstruction of tubules by renal crystals. In a very recent *in vitro* study, Asami et al.[21] prepared the melamine-urate complex by using laser desorption and supersonic-jet expansion techniques, which is nonplanar and combined *via* extremely robust multiple hydrogen-bonds. But whether the melamine-urate complex is formed *in vivo* remains to be determined.

The baseline uric acid levels as well as renal function indicators varied considerably among rats, indicating that direct comparisons based on absolute concentrations might introduce bias. Therefore, we compared the mean differences from baseline, instead of absolute concentrations for uric acid and renal function indicators. The results demonstrated that some renal function indicators varied in the control group during the 4 d of the experimental protocol. This maybe due to frequent blood collection as well as the differences between the two batches of rats we used.

In conclusion, our findings suggest the need to reconsider the previous interpretation from animal studies that human melamine toxicity is low, due to the differences in uric acid metabolism between animals and humans. Our findings also suggest a need to reconsider the notion that the toxicity of melamine occurs only in the presence of CYA[22].

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AUTHOR CONTRIBUTIONS

LIU Jian Meng had full access to all of the data and takes responsibility for the integrity of the data. Study concept: LIU Jian Meng and LI Hong Tian. Study design: LIU Jian Meng, WANG Lin Lin, and LI Hong Tian. Experiments: ZHANG Long and WANG Pei Xin. Analysis and interpretation of data: LIU Jian

Meng, ZHANG Long, and LI Hong Tian. Drafting of the manuscript: ZHANG Long, LI Hong Tian, LIU Jian Meng, and WANG Lin Lin. Critical revision of the manuscript for important intellectual content: TRASANDE Leonardo, TRACHTMAN Howard, LIU Jian Meng, LI Hong Tian, and ZHANG Long. Obtained funding: LIU Jian Meng.

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REFERENCES

1. Liu JM, Ren A, Yang Le, et al. Urinary tract abnormalities in Chinese rural children who consumed melamine-contaminated dairy products: a population-based screening and follow-up study. CMAJ, 2010; 182, 439-43.
2. Sun N, Y. ShenY, He LJ. Histopathological features of the kidney after acute renal failure from melamine. N Engl J Med, 2010; 362, 662-4.
3. Xin H, Stone R. Tainted milk scandal. Chinese probe unmasks high-tech adulteration with melamine. Science, 2008; 322, 1310-1.
4. Zhang X, Bai J, Ma P, et al. Melamine-induced infant urinary calculi: a report on 24 cases and a 1-year follow-up. Urol Res, 2010; 38, 391-5.
5. Coca SG, Yusuf B, Shlipak MG, et al. Long-term risk of mortality and other adverse outcomes after acute kidney injury: a systematic review and meta-analysis. Am J Kidney Dis, 2009; 53, 961-73.
6. Wedeen RP. Occupational and environmental renal disease. Semin Nephrol, 1997; 17, 46-53.
7. Wang L, Ding XM, Zhang KY, et al. Toxicity of cyanuric acid to broilers on hepatic and renal health with and without melamine. Hum ExpToxicol, 2012; 31, 166-73.
8. World Health Organization, Melamine and cyanuric acid: toxicity, preliminary risk assessment and guidance on levels in food. World Health Organization, 2008; 25.
9. DobsonRL, Motlagh S, Quijano M, et al. Identification and characterization of toxicity of contaminants in pet food leading to an outbreak of renal toxicity in cats and dogs. ToxicolSci, 2008; 106, 251-62.
10. Zheng X, Zhao A, Xie G, et al. Melamine-induced renal toxicity is mediated by the gut microbiota. Sci Transl Med, 2013; 5, 172ra22.
11. World Health Organization, Toxicological and health aspects of melamine and cyanuric acid: report of a WHO expert meeting in collaboration with FAO, supported by Health Canada, Ottawa, Canada, 1-4 December 2008. 2009.
12. Alvarez-Lario B, Macarron-Vicente J. Uric acid and evolution. Rheumatology (Oxford), 2010; 49, 2010-5.
13. Wei C, Moller CC, Altintas MM, et al. Modification of kidney barrier function by the urokinase receptor. Nat Med, 2008; 14; 55-63.
14. Eraranta A, Kurra V, Tahvanainen AM, et al. Oxonic acid-induced hyperuricemia elevates plasma aldosterone in experimental renal insufficiency. J Hypertens, 2008; 26, 1661-8.
15. Fridovich I. The competitive inhibition of uricase by oxonate and by related derivatives of s-triazines.J BiolChem, 1965; 240, 2491-4.
16. Iwaki K,Yonetani Y. Hyperuricemic effects of cholinergic agents in rats. Jpn J Pharmacol, 1982; 32, 343-9.
17. Yang F, Mao Y, Zhang X, et al. LC-MS/MS method for the determination of melamine in rat plasma: toxicokinetic study in Sprague-Dawley rats. J Sep Sci, 2009; 32, 2974-8.
18. Preitner F, Pimentel A, Metref S, et al. No development of hypertension in the hyperuricemic liver-Glut9 knockout mouse. Kidney Int, 2015.
19. Chang H, Shi X, Shen W, et al. Characterization of melamine-associated urinary stones in children with consumption of melamine-contaminated infant formula. Clin Chim Acta, 2012; 413, 985-91.
20. National Toxicology Program. NTP Carcinogenesis Bioassay of Melamine (CAS No. 108-78-1) in F344/N Rats and B6C3F1 Mice (Feed Study). Natl Toxicol Program Tech Rep Ser, 1983; 245, 1-171.
21. Asami H, Saigusa H. Multiple hydrogen-bonding interactions of uric acid/9-methyluric acid with melamine identified by infrared spectroscopy. J Phys Chem B, 2014; 118, 4851-7.
22. Hassani S, Tavakoli F, Amini M, et al. Occurrence of melamine contamination in powder and liquid milk in market of Iran. Food AdditContam Part A Chem Anal Control Expo Risk Assess, 2013; 30, 413-20.

Table S1. The Effect of Oxo Injection on Uric acid Levels by Dosing Groups (μmol/L)

Time (h)	Day 1				Day 4			
	0 mg/kg	200 mg/kg	400 mg/kg	600 mg/kg	0 mg/kg	200 mg/kg	400 mg/kg	600 mg/kg
1	10.86±12.16	60.69±9.89	101.26±28.16*	72.3±17.49*	31±15.26	99.01±16.53	72.73±20.78	82.43±9.8
2	−25.27±6.17	43.89±10.93	101.44±19.73*	130.55±9.19*	16.8±17.77	82.32±37.21	102.44±17.94	92.11±39.23
3	−30.26±8.64	−2.27±9.34	76.94±21.28*	113.15±19.47*	10.17±18.72	38.39±19.25	44.6±13.4	110.83±23.33*
4	−20.23±23.43	−49.38±14.82	−29.07±14.85	19.94±19.2*	4.42±20.25	−0.94±19.31	−4.89±7.09	66.07±21.81
5	−75.6±6.25	−82.05±10.36	−65.41±11.14	−29.32±14.66*	−19.08±17.88	−19.8±16.45	−27.28±12.01	16.88±16.88

Note. Data are presented as *x*±*sx* differences in uric acid levels from baseline for each time points. $^{*}P<0.05$.