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The recovery of bladder epithelial hyperplasia caused by a melamine diet-induced bladder calculus in mice

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ABSTRACT

Applying a model of bladder epithelial hyperplasia (BEH) caused by melamine-induced bladder calculus (BC), the recovery of BEH after melamine withdrawal was investigated. One experiment, comprising untreated, melamine and recovery groups, was conducted in Balb/c mice. Each group included 4 subgroups. Mice were fed normal-diet in untreated or a melamine-diet in other groups. The melamine-diet was then substituted with normal-diet in recovery group. Both of BC and BEH were observed after 14 and 56 days of melamine-diet. The BC is relatively uniform at the same melamine-diet durations. The BEH was diffuse with many mitotic figures, 4–7 rows of nuclei, and well-defined umbrella/intermediate cells. No marked differences in BEH degree were observed in the two different melamine-diet durations. On 4–42 days after melamine withdrawal, BC was not found, as the progressive regression with complete regression of BEH was observed, along with well-defined ageing/apoptotic cells in the superficial regions of BEH regression tissue. Conclusion, the melamine-induced BEH is relatively uniform, may be self-limiting in rows of nuclei, and can return to normal. Melamine withdrawal duration is critical for the BEH regression. Tissue of the BEH and its regression is ideal for exploring the renewal as well as growth biology of mammalian urothelium.

1. Introduction

Following a large outbreak of infant urolithiasis caused by melamine-contaminated milk products in China in 2008 (Chen, 2009; Wu et al., 2009), it is well-known that high melamine dose diets can quickly cause urolith and urothelial hyperplasia (Xu et al., 2011; Ren et al., 2012; Wu and Zhang, 2013).

After melamine withdrawal, regardless of treatment status, the uroliths caused by melamine usually disappear within a few days in affected mice (Ren et al., 2012) and infants (Chen, 2009; Dalal and Goldfarb, 2011; Sun et al., 2009; Zhang et al., 2009; Zhu et al., 2009), although the disappearance duration of the urolith increases slightly as the urolith size and/or density increases (Ren et al., 2012; Shen et al., 2011). However, to date, it has been unclear if the degree of urothelial hyperplasia markedly increases with increasing melamine intake duration or urolith size/density, if the hyperplastic urothelium (Heck and Tyl, 1985; Mast et al., 1983; Melnick et al., 1984; Puschner and Reimschuessel, 2011; Ren et al., 2012; Wu and Zhang, 2013; Xu et al., 2011) can return to normal following the rapid disappearance of the urolith after melamine withdrawal, and if the time to achieve complete regression (i.e., recovery) for the epithelial hyperplasia is remarkably increased with an increase in melamine intake duration or urolith size/density or urolith disappearance duration.

In essence, the urothelial hyperplasia is an adaptive response of urothelium against urolith stimulation (Xu et al., 2011), and can generally return to normal *via* its re-adaptation or regression following the disappearance of the uroliths after melamine withdrawal, but urothelial hyperplasia and its regression are easily ignored and difficult to monitor in infants, and also are major concerns of parents and doctors of infants with urolithiasis. Moreover, epithelial hyperplasia with its regression, as a basic biological event, is also important in epithelial cell biology. Therefore, the histological characterization of urothelial hyperplasia and its regression is worthwhile to further investigate.

In this study, a model of bladder epithelial hyperplasia (BEH) caused by melamine-induced bladder calculus (BC) developed by our group (Xu et al., 2011; Ren et al., 2012) was used, and the recovery of BEH after melamine withdrawal were evaluated.

Abbreviations: BC, bladder calculus; BEH, bladder epithelial hyperplasia; h, hours; SPF, specific-pathogen free; bw, body weight; F, female; M, male; w, weeks old.

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2. Materials and methods

2.1. Chemicals

Melamine [2,4,6-triamino-*s*-triazine, CAS No. 108-78-1] was purchased from Xingfu Fine Chemicals Research Institute, Beijing, China (Batch No. 20060220; analytical pure, purity 99.5%; http://www.fubide.com).

2.2. Animals and diets

The experimental protocol was approved by the Laboratory Animal Administration Committee of Xi'an Jiaotong University and performed according to the University Guidelines for Animal Experimentation. Balb/c mice (SPF) were obtained from and housed in the University Laboratory Animal Centre. A normal diet (normal-diet) (GB14924.3-2010, China) (http://www.foodmate.net) or a 9373 ppm melamine diet (melamine-diet) were used.

This melamine dose has been calculated based on a mathematical equation using 3 basic parameters {golden section [a well-known preferred proportion (0.618)], the 50% lethal dose (LD50) of mouse oral melamine [4550 mg/kg body weight (bw)], and mouse average daily consumption from diet (300 g diet/kg bw)} under two hypotheses {[1] 50% lethal dose may be considered as the max daily dose and [2] the most appropriate daily dose of dietary melamine intake required for BC formation may be close to the LD50's golden section} (Xu et al., 2011). This 9373 ppm melamine dose has been tested for the rapid and stable formation of BC and BEH in our previous experiments (Xu et al., 2011; Ren et al., 2012), which was better than other two doses [9842 (i.e., 9373 × 105%) and 8904 (i.e., 9373 × 95%) ppm] (Xu et al., 2011). Thus this melamine dose was used in the current study.

The temperature range in the animal rooms was from 18 °C to 22 °C, while the relative humidity was from 40% to 60%, the light/dark cycle was 12 hour (h)/12 h, and sterile water (pH 6.0) was provided ad libitum.

2.3. Experimental design

One experiment was conducted in 122 Balb/c mice [60 at 5–6 weeks of age (w) and 13–16 g of body weight (bw), and 62 at 3–4 w and 8–12 g of bw]. The mice were divided into 3 groups: untreated, melamine and recovery groups, with 4 subgroups in each group (Table 1). The mice were fed with the normal-diet in the untreated groups and the 9373 ppm melamine-diet in the melamine and recovery groups for the designated durations, and the first day was set as experiment-day 1. The melamine-diet was then substituted with the normal-diet in 4 subgroups of recovery groups (i.e., melamine withdrawal), and the first day after melamine withdrawal was set as recovery-day 1.

To evaluate the effects of melamine intake duration (or BC size/density) on the degree of BEH, and the effects of melamine intake duration and/or the duration of time after melamine withdrawal (i.e., recovery duration) on the BEH regression, the 1 and 2 recovery subgroups with the same melamine-diet duration [14 days, a suitable duration for the stable establishment of this BC and BEH model system (Xu et al., 2011; Ren et al., 2012)] and the near recovery duration (4 and 8 days after melamine withdrawal, respectively), and the 3 and 4 recovery subgroups with the greatly different melamine intake duration (14 and 56 days, respectively) and the same recovery duration (42 days after melamine withdrawal), as well as the corresponding subgroups in untreated and melamine groups, were designed.

For the comparison of proper subgroups, the 5–6 week-old mice in the 1 and 2 subgroups, and the 3–4 week-old mice in the 3 and 4 subgroups, were used (Table 1): (1) untreated group, including subgroups 1–3 ($n = 10$ in each subgroup, 14-day normal-diet), and 4 ($n = 8$, 56-day normal-diet); (2) melamine group, including subgroups 1–3 ($n = 10$ in each subgroup, 14-day melamine-diet), and 4 ($n = 8$, 56-day melamine-diet); and (3) recovery group, including subgroups 1–3 ($n = 10$ in each subgroup, 4, 8 and 42 days after 14-day melamine-diet, respectively), and 4 ($n = 16$, 42 days after 56-day melamine-diet). The ratio of females (F) to males (M) in each subgroup was 1:1.

The general state of each mouse was observed daily. The incidence and morphology of BC and the histomorphology of bladder epithelium were observed at the designated duration in the mice of each subgroup.

2.4. Experiment

In this experiment, the incidence and morphology of bladder calculus and the histomorphology of bladder epithelium were investigated.

On the designated durations, the mice were anesthetized by peritoneal injection of 20% urethane. Then, the abdominal cavity of each mouse was opened along the anterior midline of abdominal wall, and the urine samples were collected by bladder puncture *in situ* and pH of urine was measured with pH paper (indicator paper, Q/GHSC, Shanghai SSS Reagent Co., Ltd., Shanghai, China). The bladder of each mouse was opened *in situ* along the anterior midline and examined, and all calculi were removed. After air drying, the calculi were stored at room temperature. Subsequently, gross images of the calculi were recorded by camera (Sony DSLR-A-100). And next, the mice were sacrificed, and the bladder tissue of each mouse was immediately immersed in 10% neutral buffered formalin, routinely processed, mounted in paraffin, sectioned at 4 μm, and stained with hematoxylin–eosin (HE). All sections were systematically and comparatively observed by 2 pathologists.

The degree of each mouse BEH was assessed according to the extent and growth tendency of BEH, as well as the rows of nuclei in the hyperplastic bladder epithelium. The progression of each mouse BEH regression in recovery group was assessed, based primarily on the previously reported criteria (Ren et al., 2012) except that the description 'bladder epithelial layers' was replaced with the description 'rows of nuclei' on the cut surface (perpendicular to basal membrane) of hyperplastic bladder epithelium in the present criteria. The progression includes regressive tendency (RT, showed by a marked decrease in the size of the epithelial cells and nuclei and nucleoli, the marked decrease or absence of mitotic figures, and 4–7 rows of nuclei), regression (R, exhibited by a marked decrease in the size of the epithelial cells and nuclei and nucleoli, the absence of mitotic figures, and 3–5 rows of nuclei), and significant regression (SR, displayed by a marked decrease in the size of the epithelial cells and nuclei and nucleoli, the absence of mitotic figures, and nearly normal rows or 2–4 rows of nuclei) (Ren et al., 2012), as well as complete regression [CR, i.e., recovery, presented by the histomorphology of normal bladder epithelium (2–3 rows of nuclei)]. The histological images of the bladder epithelium were captured using an Olympus BX51-DP71 system.

3. Results

In this study, there were no deaths of the mice in any of the experimental groups.

In the untreated group (Table 1), no abnormalities or signs of BC or BEH were observed in any of the mice. The pH range of the urine ranged from 5.8 to 6.0. The bladder epithelium was 2–3 rows of nuclei and composed of 3 cell types: umbrella, intermediate and

Table 1
Bladder epithelial hyperplasia and its regression and the related data.

Group	No. of mice	IW/BW (g)	ED/RD (days)	Incidence of BC	Incidence of BEH	No. of mice with RT/R/SR/CR	Urine pH range
Untreated							
1	10	5–6/13–16	14/0	0/10	0/10		5.8–6.0
2	10	5–6/13–16	14/0	0/10	0/10		5.8–6.0
3	10	3–4/8–12	14/0	0/10	0/10		5.8–6.0
4	8	3–4/8–12	56/0	0/8	0/8		5.8–6.0
Melamine							
1	10	5–6/13–16	14/0	10/10	10/10		5.0–5.2
2	10	5–6/13–16	14/0	10/10	10/10		5.0–5.2
3	10	3–4/8–12	14/0	10/10	10/10		5.0–5.2
4	8	3–4/8–12	56/0	8/8	8/8		5.0–5.2
Recovery							
1	10	5–6/13–16	14/4	0/10		6/4/0/0	5.8–6.0
2	10	5–6/13–16	14/8	0/10		0/6/4/0	5.8–6.0
3	10	3–4/8–12	14/42	0/10		0/0/6/4	5.8–6.0
4	16	3–4/8–12	56/42	0/16		0/0/10/6	5.8–6.0

Abbreviations: IW/BW (g), as initial age as weeks and initial body weight (grams); ED/RD, experiment-days/recovery-days (i.e., days after melamine withdrawal); BC, bladder calculus; BEH, bladder epithelial hyperplasia; RT/R/SR/CR, regressive tendency/regression/significant regression/complete regression phenotypes of BEH.