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Time Course of Urinary Metallothionein Excretion in Rats Exposed to Cadmium

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Abstract: Cadmium chloride was administered to the rats, subcutaneously, at the doses of 0, 0.5, 1.0 and 2.0 mg Cd kg⁻¹ body weight, 5 times/week up to 3 weeks. The levels of cadmium, metallothionein and N-acetyl-β-D-glucosaminidase were determined in urine within the exposed period. Both urinary cadmium and metallothionein increased dose-time-dependently upon cadmium exposure. The continuously mild increasing excretion of urinary metallothionein was observed in the rats exposed to 1.0 mg Cd kg⁻¹, starting from the 10th day after cadmium exposure. The excretion of urinary metallothionein started increasing from one week after cadmium exposure and further significant more excretion after two weeks, when NAGuria appeared, in the group exposed to 2.0 mg Cd kg⁻¹. There was a similar pattern for urinary metallothionein excretion in both genders of rats. In this study it has been well demonstrated that there are two phases of increasing excretion of urinary metallothionein, i.e., the first phase, which could be related to overflow of new synthesized metallothionein in kidney and the second phase, which was the result of cadmium induced renal tubular dysfunction.

Key words: Time course, cadmium, urinary cadmium, urinary metallothionein, urinary N-acetyl-β-D-glucosaminidase, renal tubular dysfunction

INTRODUCTION

Cadmium (Cd) is an important environmental pollutant, which causes a number of adverse health effects. Chronic exposure to cadmium results in its progressive accumulation in various tissues, particularly in kidney and can lead to renal tubular dysfunction, characterized by low molecular weight proteinuria both in experimental animals and human.

Metallothioneins (MTs), a unique low molecular weight intracellular protein rich in cysteine, is synthesized mainly in liver and kidney after exposure to cadmium. It is well known that metallothionein plays a crucial role in cadmium kinetics and toxicity (Jin *et al.*, 1998; Nordberg and Nordberg, 2000). Metallothionein, induced in tissues, appears in blood and urine upon continued cadmium exposure. Indeed, the excreted amounts of metallothionein in urine were found to depend on the cadmium levels in both liver and kidney of Cd-injected rats (Tohyama *et al.*, 1981). Numerous studies had shown that the levels of urinary MT significantly correlated with those of urinary Cd both in Cd-exposed population and Cd-injected rats (Shaikh and Tohyama, 1984; Kawada *et al.*, 1990; Roels *et al.*, 1983). Moreover, the subjects with abnormal renal function excreted higher amounts of urinary MT than those with normal renal function (Shaikh *et al.*, 1987; Chen *et al.*, 2006). Tohyama *et al.* (1988) described that the excretion of urinary MT was closely associated with the indices of renal dysfunction, such as β₂-microglobulin in people living in a Cd-polluted area. Kawada *et al.* (1990) also concluded that there was a pronounced correlation between the level of

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urinary MT and urinary N-acetyl- β -D-glucosaminidase (NAG) in workers occupational exposed to cadmium. These studies indicated that elevated urinary MT excretion could not only serve as an index of excessive cadmium exposure but also of renal dysfunction caused by chronic exposure to this metal. Although urinary MT had been a specific biomarker of cadmium nephrotoxicity, the detailed time course of metallothionein excretion in urine rarely had been reported. Metallothionein in urine may be contributed to desquamation of the renal tubular epithelial cells as well as by direct renal secretion into the urine in the early phase of cadmium administration (Tohyama *et al.*, 1981). A decreased tubular reabsorption also be responsible for an increased excretion of urinary MT (Lauwerys *et al.*, 1984). In this study, we aimed to observe the time course of urinary MT excretion in rats exposed to cadmium and confirm the mechanism of urinary MT excretion based on the relationship between urinary MT excretion and development of cadmium nephrotoxicity.

MATERIALS AND METHODS

Animal Treatment

Forty-eight Sprague-Dawley rats, weighing 180-220 g, were purchased from the Animal Research Center of Fudan University. Prior to the experiment all rats were acclimatized to the animal facilities for 1 week. The animals were maintained under controlled temperature condition ($22\pm 2^\circ\text{C}$) and with a 12 h day-night cycle. Food and tap water were provided *ad libitum* during the entire experiment. After the acclimatization, the rats were divided into 4 groups of 12 rats each (male/female = 1:1). Group A constituted control animals which received subcutaneous injection of 1.0 mL kg^{-1} body weight normal saline. Group B, C and D were subcutaneously injected with CdCl_2 at the doses of 0.5, 1.0, 2.0 mg Cd kg^{-1} body weight, respectively, 5 days/week, for up to 3 weeks.

At specified intervals the rats were individually transferred to metabolic cages and the urine of each rat was collected for 24 h in a vessel kept in an ice-bath. Urine samples were collected at the following time period of the experiment: 0, 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 days, respectively and urinary volume were recorded. The urine samples were frozen and stored at -70°C until analyzed. All rats were sacrificed on the 21st day.

Analysis

Cadmium concentration in urine was measured by flame atomic absorption spectrometry (FAAS) after digestion of the samples with an equal volume of concentrated nitric acid. The levels of urinary MT were determined by an enzyme-linked immunosorbent assay (ELISA) as modified by Chen *et al.* (2006) and Jin *et al.* (1992). The levels of urinary NAG were analyzed as previously described by Tucker *et al.* (1975). The creatinine (Crea) concentration was measured by the Jaffe reaction method and was used to adjust other parameters in urine.

Statistical Methods

Analyses were undertaken using SPSS 11.5 statistical analysis software. The urinary Cd, MT and NAG concentration were normalized by logarithmic transformation, which were expressed in terms of geometric means (GM). One-way analysis of variance (ANOVA) was used and the criteria of significance was set at $p < 0.05$.

RESULTS

Body and Organ Weight

Figure 1a and b show the average body weight of the male and female rats in each group, respectively, which increased gradually with the duration of the study. For males the body weight of

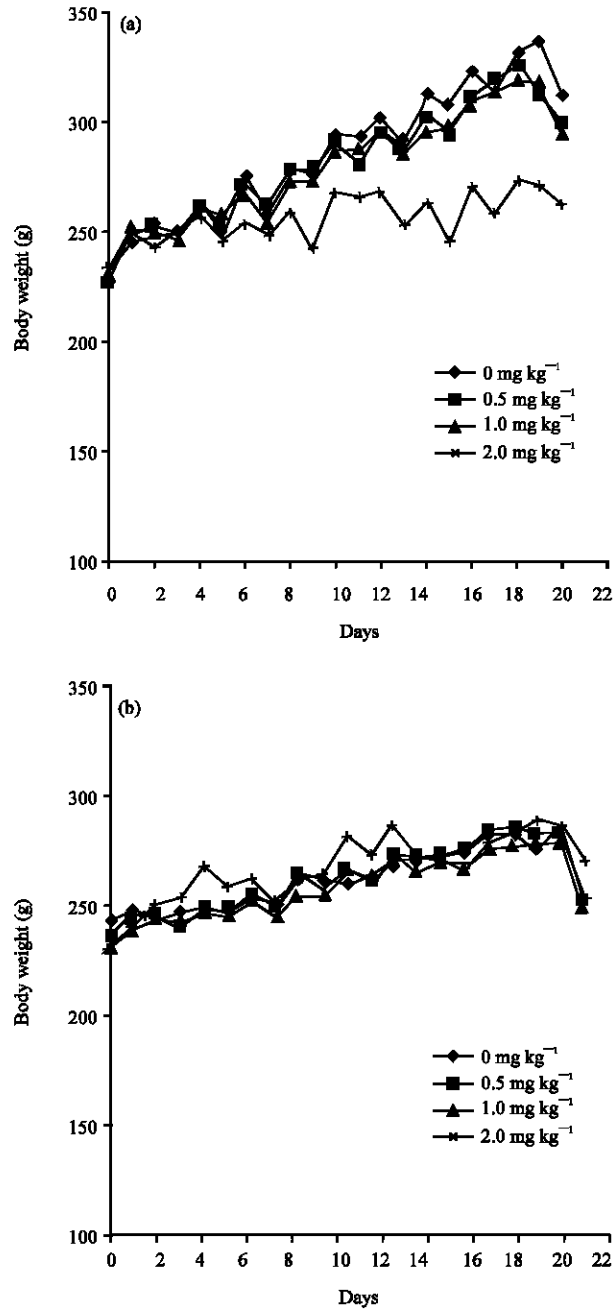


Fig. 1: The growth curves for (a) male and (b) female rats

the rats exposed to 0.5 and 1.0 mg Cd kg⁻¹ did not show any difference compared to the control group throughout the experiment. But in the dose of 2.0 mg Cd kg⁻¹ group the body weight of the rats were significantly lower than the controls from day 6 ($p < 0.05$) and the difference become progressively larger at all subsequent days. However, for female rats, the effect on the body weight of cadmium was not obvious like that of males. There was no difference between control and Cd-treated groups all the time.

Table 1: The ratio of organ weight ($\times 1000$) to body weight in male and female rats

Dose (Mg kg ⁻¹ b.w)	Male			Female	
	No	Liver	Kidney	Liver	Kidney
0	6	32.13 \pm 0.45	6.97 \pm 1.31	30.57 \pm 2.15	7.58 \pm 0.46
0.5	6	32.55 \pm 1.98	8.21 \pm 0.71	33.65 \pm 3.32	7.85 \pm 1.31
1.0	6	37.18 \pm 4.18#	8.69 \pm 0.54*	37.78 \pm 4.10#	8.81 \pm 0.57*
2.0	6	44.88 \pm 3.44#	11.61 \pm 3.24#	50.62 \pm 3.57#	10.06 \pm 0.73#

Values are Mean \pm Standard deviation, * and # represent the statistical difference at $p < 0.05$ and $p < 0.01$ levels

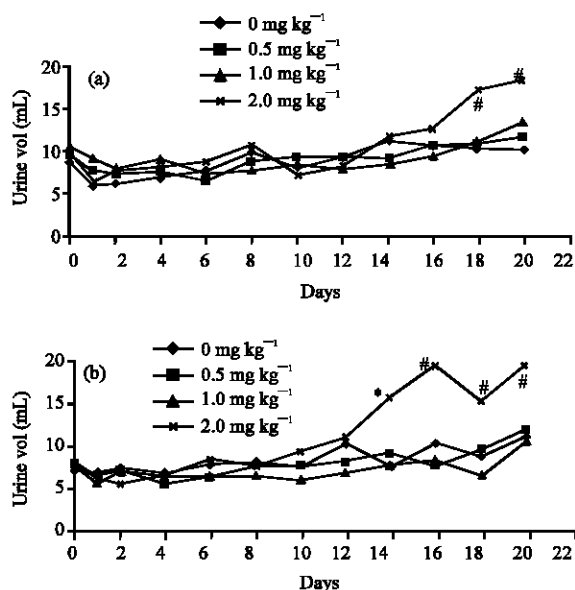


Fig. 2: The time-courses of the levels of urinary volume in (a) male and (b) female rats, * and # represent the statistical difference at $p < 0.05$ and $p < 0.01$ levels

The livers and kidneys from Cd-treated rats were noticeably enlarged due to the damages caused by cadmium. In both male and female rats, the ratio of liver and kidney weight to body weight increased dose-dependently, which were significantly higher in the groups with the dose of 1.0 and 2.0 mg Cd kg⁻¹ ($p < 0.05$) (Table 1).

The Time Course of the Urinary Volume in Rats

For males, the urinary volume of the rats exposed to 0.5 and 1.0 mg Cd kg⁻¹ did not change significantly throughout the experiment. While in the dose of 2.0 mg Cd kg⁻¹ group the urinary volume started increasing from day 14 and was significantly elevated on day 18 and day 20 (Fig. 2a). As to the female rats, the changes of the urinary volume were similar to those of males. Especially in the dose of 2.0 mg Cd kg⁻¹ group the urinary volume prominently increased from day 14 until the end of experiment (Fig. 2b).

The Time-courses of the Urinary Cd in Rats

For males, the urinary excretion of cadmium in the dose of 0.5 mg Cd kg⁻¹ group was altered only slightly. In the dose of 1.0 mg Cd kg⁻¹ group, the urinary Cd concentration increased gradually up to day 12, when there was significant difference compared to the control level ($p < 0.05$). Urinary Cd in the group with the dose of 2.0 mg Cd kg⁻¹ was pronounced elevated from day 4 ($p < 0.05$) and then increased linearly to the end of the experiment, of which the maximum level was observed on day 18,

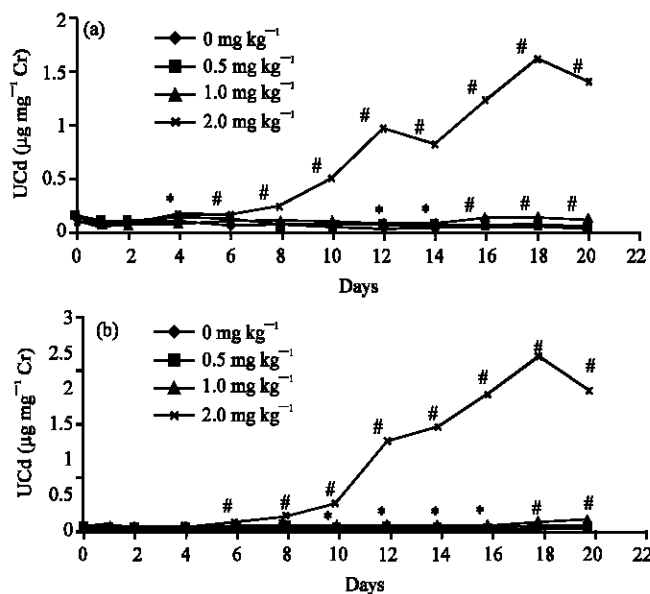


Fig. 3: The time-courses of the levels of urinary Cd in (a) male and (b) female rats, * and # represent the statistical difference at $p < 0.05$ and $p < 0.01$ levels

approximate 36 times higher than the control level (Fig. 3a). The time-course of urinary Cd in the Cd-treated groups of female rats showed a similar increasing trend whereas the levels of urinary Cd were noted on day 6 and day 8 in the groups with the dose of 2.0 and 1.0 mg Cd kg⁻¹, respectively ($p < 0.05$) (Fig. 3b).

The Time-courses of the Urinary MT in Rats

As shown in Fig 4a and b, the urinary MT levels in the control groups of the males and females did not change significantly after injection of saline. The urinary MT concentrations increased at different rates in the different Cd-treated groups. For male rats, there was minor change of the urinary MT in the dose of 0.5 mg Cd kg⁻¹ group only appearing statistically difference on day 18, 20 ($p < 0.05$), the last two days of the experiment. The urinary excretion of metallothionein in the dose of 1.0 mg Cd kg⁻¹ group started to increase on day 10 ($p < 0.05$) and continue to increase with time until day 20. Rats in the dose of 2.0 mg Cd kg⁻¹ group excreted significantly high level of urinary MT in comparison to the control from day 6 ($p < 0.05$) and then further increased until day 14, reaching a peak value of 142.30 $\mu\text{g mg}^{-1}$ creatinine. Thereafter, the metallothionein concentration decreased slightly and leveled off to the end of experiment. Change processes of the urinary MT in the female rats showed similar patterns to those of males except that the urinary MT levels in the dose of 0.5, 1.0 and 2.0 mg Cd kg⁻¹ groups were prominently elevated from day 16, day 12 and day 6, respectively ($p < 0.05$). Moreover, in the dose of 2.0 mg Cd kg⁻¹ group there was a sharp peak of 250.55 $\mu\text{g mg}^{-1}$ creatinine in the urinary MT concentration on day 14, which was 25 times higher than the control level.

The Time-Courses of the Urinary NAG in Rats

In our study, no significant increase in urinary NAG were observed in the male rats exposed to 0.5 and 1.0 mg Cd kg⁻¹ compared to the control levels throughout the experiment. The excretion of urinary NAG in the male rats exposed to 2.0 mg Cd kg⁻¹ increased slowly, remaining within control limit to day 14, when an increase of urinary NAG activity occurred that was significant higher than

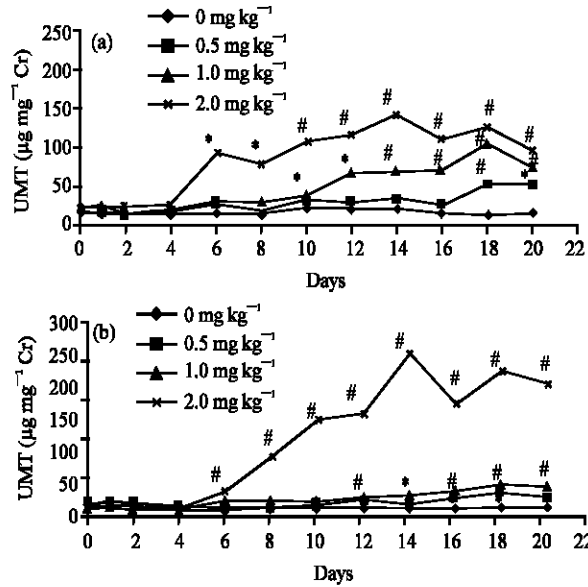


Fig. 4: The time-courses of the levels of urinary MT in (a) male and (b) female rats. *and # represent the statistical difference at $p<0.05$ and $p<0.01$ levels

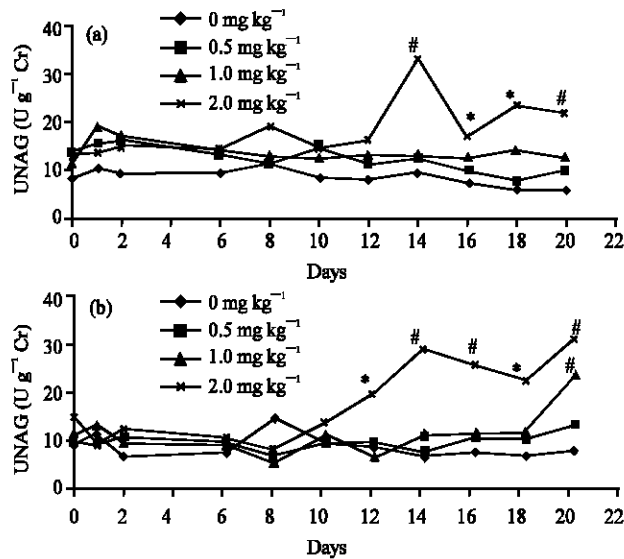


Fig. 5: The time-courses of the levels of urinary NAG in (a) male and (b) female rats, * and # represent the statistical difference at $p<0.05$ and $p<0.01$ levels

the control value ($p<0.05$) and thereafter it reached plateau till the end of the experiment. Although the urinary NAG in the female rats resembled that of the males, there were still a few differences between the genders. First in the female rats exposed to $1.0 \text{ mg Cd kg}^{-1}$ there was a great excretion of urinary NAG in comparison to the control on the last day of the experiment ($p<0.05$). Second the urinary NAG in the dose of $2.0 \text{ mg Cd kg}^{-1}$ group began to increase significantly on day 12 ($p<0.05$), two days earlier than the male rats (Fig. 5a and b).

DISCUSSION

In the present study, we dynamically observed the time course of urinary MT excretion in rats exposed to cadmium. Continued exposure to cadmium could cause renal tubular dysfunction both in experimental animals and human. To evaluate renal damage, urinary NAG has been extensively used as an early sign of renal tubular dysfunction caused by cadmium (Nogawa *et al.*, 1986; Kawada *et al.*, 1989).

Metallothionein, initially synthesized in liver, binds the cadmium and is later released into the bloodstream. The circulating Cd-MT complex, due to its low molecular weight, is efficiently filtered through the renal glomerulus. Although most of the filtered metallothionein is reabsorbed by the proximal tubules, a small part of the protein is excreted in urine in a concentration-dependent manner (Sugihira *et al.*, 1986). After reabsorption by kidney, the metallothionein is degraded in the lysosomes and free cadmium is released to react with sensitive sites in the cell (Fowler and Nordberg, 1978). With the duration of exposure to cadmium, renal damage will occur when a sufficient amount of non-MT-bound cadmium has accumulated in the renal tissue (Chang *et al.*, 1980). Subsequently, the excretion of metallothionein like other low molecular weight protein (i.e., β_2 -microglobulin, retinol-binding protein, etc.), significantly increases in urine arise from cadmium induced renal tubular dysfunction characterized by decreased renal tubular reabsorption. In our study, the urinary excretion of metallothionein started increasing mildly around day 10 and day 18 in the rats exposed to 1.0 and 0.5 mg Cd kg⁻¹, respectively. But no significant difference of the urinary NAG and urinary volume were found in these two groups throughout the experiment. However, in the group exposed to 2.0 mg Cd kg⁻¹, the urinary excretion of metallothionein started increasing within one week after exposure to cadmium and then the levels of urinary MT were further significantly elevated in the second week, which reached a peak value on day 14, when the levels of urinary NAG were significantly increased and followed by marked increase in the urinary volume, both of which indicated that renal tubular dysfunction appeared. In this study it has been clearly demonstrated and confirmed that there are two phases of increasing excretion of urinary MT after continued exposure to cadmium. During the first phase, before appearance of renal damage, the urinary MT increased moderately which was mostly related to direct overflow of new synthesized metallothionein upon cadmium exposure. With the duration of exposure to cadmium, large amount of metallothionein was excreted in urine time-dependently due to the decreased capacity of renal tubular reabsorption, which was in accordance with the previous study (Sugihira *et al.*, 1986) suggesting that the second phase of urinary MT excretion resulted from Cd-induced renal tubular dysfunction.

In this study the time course of urinary MT elucidated the relationship among cadmium exposure, urinary MT and cadmium nephrotoxicity. Generally, urinary Cd, β_2 -microglobulin and retinol-binding protein have been the biological indicators of cadmium exposure. However, these parameters are not specific indices, which could be affected by certain other conditions (Kido *et al.*, 1991). On the contrary, metallothionein is induced upon cadmium exposure and is consider to be a specific and sensitive indicator of cadmium nephrotoxicity (Shaikh *et al.*, 1990; Nordberg *et al.*, 1982). According to our results, in the first phase of urinary MT excretion, the levels of urinary MT started increasing on day 6 and behaved together with urinary Cd in the rats exposed to 2.0 mg Cd kg⁻¹. While in the second phase of urinary MT excretion, approximately 20-fold increase in urinary MT was accompanied with the elevated excretion of urinary NAG, which was used as the marker of renal damage. These results indicated that the urinary MT level could be an indicator not only of cadmium exposure in the early phase but also of the renal tubular damage caused by long-term exposure to cadmium.

In conclusion, present study depicted the time course of urinary MT excretion in rats exposed to cadmium, indicating that there were two phases in the excretion process of urinary MT and each phase probably reflected the intensity of cadmium nephrotoxicity.

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