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THE LATE AND PERSISTENT PATHOGENIC EFFECTS OF CADMIUM AT VERY LOW LEVELS ON THE KIDNEY OF RATS

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□ Cadmium (Cd) is an important nephrotoxic pollutant. To examine late effects on the kidney of individuals previously exposed to chronic Cd at very low levels, male Wistar rats were given 20 nmol/kg i.p. injections of Cd every other day for 4 weeks. At the 20th, 28th, 36th, 44th and 52nd week of the study, renal metal accumulation, morphology and function were examined. Immunochemical staining was performed to detect renal 3-nitrotyrosine (3-NT) accumulation, metallothionein (MT) expression, cell proliferation and global DNA methylation. Results showed that renal Cd concentration and MT expression along with 3-NT accumulation were significantly higher in the Cd group than that in the control. Histopathologically renal tubule damage at the early stage and hyperplasia at the late stage were observed in the Cd group. Renal fibrosis in glomeruli was evident in the Cd group, particularly at the late stage of the study. Immunoreactivity of global DNA methylation was markedly diminished in the Cd group at both 20th and 52nd weeks. These results suggest that previous exposure to chronic Cd at very low level induced persistent damaging effects on the kidney along with increases in cell proliferation and global DNA hypomethylation.

Key Words: Cadmium; renal toxicity; persistent effect; renal dysfunction; methylation

INTRODUCTION

Cadmium (Cd) is a nonessential metal that causes several human diseases and has been classified as a human carcinogen by the National Toxicology Program of the USA (ATSDR, 2004). Several population stud-

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ies have shown that not only occupationally Cd-exposed workers but also environmentally Cd-exposed populations can experience moderate to severe health problems (Bernard *et al.* 1992; Bernard *et al.* 1990; Jarup and Alfven 2004). Unfortunately, human contact with Cd is an inescapable consequence of human life, from both occupational and environmental sources. The environmental exposure of Cd occurs mainly from consumption of contaminated food, active and passive inhalation of tobacco smoke, and inhalation by workers in the non-ferrous metal industry. In humans and other mammals, Cd adversely affects a number of organs and tissues, with the kidney and liver as two primary target organs (Zalups and Ahmad 2003). Chronic exposure to low-level Cd has been associated with a number of pathogenic changes, such as the end-stage renal failure, early onset of diabetic renal complications, and increased cancer risk (Jarup 2002; Nakagawa and Nishijo 1996). Cd accumulates primarily in the kidney and is eliminated very slowly with a half-life of 15–20 years (Jarup 2002). In our recent study, we observed a persistent increase of hepatic cell proliferation in the rats at 44^{th} and 52^{nd} weeks after chronic exposure to Cd at low dose (Wang *et al.* 2011).

The biological effects of Cd at high doses have been extensively investigated (Moulis and Thevenod 2010). Chronic exposure of animals to Cd at low levels has been studied by several groups (Bernard *et al.* 1992; Bernard *et al.* 1990; Jarup and Alfven 2004). However, most of those studies have focused on the effects during or at the end of Cd exposure.

Recently whether the toys and jewelries containing small amount of Cd that contributes to a resource for children's Cd contamination should be banned in order to avoid the potential toxicity of Cd are debated (Mead 2010). The debate is mainly because we do not know whether exposure of the individuals to Cd at very low levels, although it does not produce significant acute toxicity, can induce a late or long-lasting effect (Mead 2010). However, there was an indirect evidence that previous smokers had increased risk of renal dysfunction compared to non-smokers (Cooper 2006). Since tobacco smoking increases systemic Cd levels, previous Cd exposure due to smoking may cause a long-lasting toxic effect.

In this study, therefore, we aimed at investigating renal response at functional, histopathological and biochemical levels in rats previously exposed to chronic Cd at very low dose (20 nmol/kg body weight). CPSC 2010's has published an acceptable daily intake level of 0.1 μg/kg body weight/day for chronic exposure (Mead 2010). We decided to examine whether there was a late or long-lasting renal effect of Cd at about 20 folds of this acceptable daily intake dose (20 nmol/kg/day ≈ 2.25 μg/kg/day), which would be the lowest level of Cd used for animal study based on our knowledge. In order to address renal effects of Cd at very low level, we decided to use i.p. injection, instead of dietary administration, of Cd to ensure the accurate dose administration without influence

of intestinal absorption difference between early and late stages for Cd ingestion. We examined renal structure and function for the Cd-treated and age-matched rats at the 20th, 28th, 36th, 44th and 52nd weeks from the beginning of the 4-week Cd-treatment.

MATERIALS AND METHODS

Animals and Treatment

Thirty-six adult male Wistar rats (6-week old), weighing 180–200g, were purchased from the Experimental Animal Center of School of Basic Medical Sciences, Jilin University (Changchun, China). Rats were fed with standard laboratory chow and tap water *ad libitum* for 1 week before the experiments was commenced. The experiments were approved by and carried out in accordance with the regulation of University Animal Experimentation Committee. These rats were housed in plastic cages with light and dark (12 h: 12 h) and an ambient temperature of 24°C ± 2°C. The rats were divided randomly into two groups (n=8), and 2 cages (4 rats/cage) were used separately for each group; (1) control group: rats were given i.p. injections of saline every other day for 4 weeks. (2) Cd treatment group: rats were given i.p. injections of Cd in saline at 20 nmol/kg every other day for 4 weeks. Cadmium chloride ($CdCl_2$; purity 99.99 %) was obtained from Sigma-Aldrich (St. Louis, MO, USA). The time at the beginning of 4-week Cd exposure was set up as time 0 of the study (Fig. 1A). From these mice, livers have already been collected and analyzed and published elsewhere (Wang *et al.* 2011). Since renal toxic effects of Cd at low doses have been extensively investigated at the end of Cd treatment (Jarup and Alfven 2004; Jarup *et al.* 2000; Uriu *et al.* 2000), our

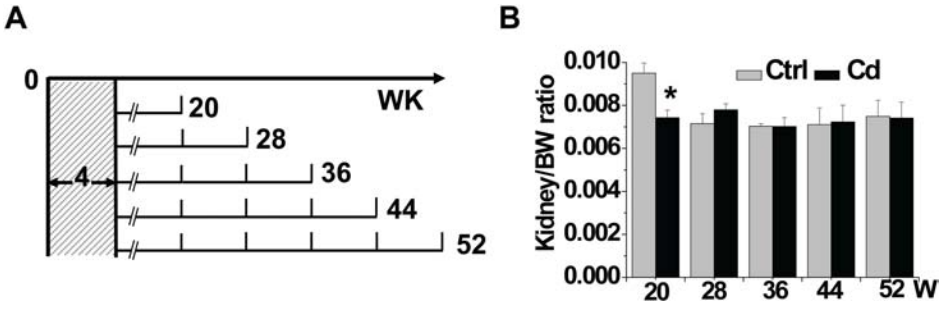

FIGURE 1. Outline of the study and relative kidney weight. (A) Outline of the animal treatment time and the time (weeks) of sacrifice. Rats were given i.p. injections of Cd in saline at 20 nmol/kg body weight every other day for 4 weeks (grey diagonal area). Age-matched control rats were given i.p. injections with same volume saline every other day for 4 weeks. The beginning of Cd injection was set as 0, and at the 20th, 28th, 36th, 44th and 52nd weeks of the study, rats were sacrificed. (B) Relative kidney weight, i.e.: the ratio of kidney weight to body weight, in each rat was analyzed since the body weight was no different between Cd group and control group (data not shown). Data was presented as mean ± SD (n=6). $^{*}P < 0.05$ vs corresponding controls.

focus here was to investigate whether there remains late or persistent effects of previous exposure to chronic Cd. Accordingly the experiments were carried out at the 20th, 28th, 36th, 44th, and 52nd weeks from the beginning of the 4-week Cd exposure, as illustrated in Fig. 1A. Since Wistar rats have a lifespan of 30 months (LaSarge and Nicolle 2009), these rats at the time of sacrifice were correspondingly 21.66% (6 weeks old + 20 weeks for the study) and 48.33% (6 weeks + 52 weeks) of their lifespan, respectively for the first and last time-points. The rats were weighed and killed using an overdosage of pentobarbital sodium at 50 mg/kg body weight i.p. (Sigma, Chicago, USA) at the time of sacrifice. Then the kidneys were removed quickly from animals, cleaned and washed in precooled saline several times, and then were portioned and stored at –80°C.

Sample Preparation for Determining Tissue Metal Concentrations

At the time of sacrifice, 100 mg of the kidney in wet weight were ground, put in scaled glass tube, and then dried in oven at 105 °C for 4 h. Tissues in the scaled tubes were digested with 0.5 ml 50% (vol/vol) HNO_3. Upon completion of digestion, the inorganic residues were dissolved in deionizing distillated water until 2 ml and the Cd concentration was measured using inductively coupled plasma mass spectrometry (ICP-MS; Agilent Technologies, Santa Clara, CA, USA). In addition to Cd, copper (Cu), zinc (Zn) and calcium (Ca) were measured. Concentrations are reported as nan- or micro- grams of metal per gram of dry weight (ng or μg/g).

Biochemical Parameters

The concentrations of blood urea nitrogen (BUN) and creatinine (Cre) in the serum were tested with ROCHE COBAS 400 full-automatic biochemistry analyzer. Kidney tissue from each rat was homogenized in 10 volumes of precooled phosphate buffered saline (pH 7.4), and the homogenate was centrifuged at 3,000 × g for 15 min to collect the supernatant for biochemical assays. Activities of superoxide dismutase (SOD) and contents of glutathione (GSH) and malondialdehyde (MDA) in the kidney were detected using corresponding commercial assay kits (Jiancheng, Nanjing, China).

Tissues for Histopathology

For pathological examination for Cd-induced kidney lesions, the tissue was fixed in 10% buffered formalin, embedded in paraffin, and sectioned at 4 μm. After deparaffinization, tissue sections were rehydrated and stained by hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) reagent. Pathological changes of glomeruli, renal tubules and interstitium were examined by light microscopy. All morphometric and semi-quantitative analysis were performed in a blinded manner by two

investigators without knowledge of the origin of the section. Histopathology for tissues was evaluated for at least 10 randomly selected non-overlapping fields of each section. Mesangial index was assessed based on at least 20 glomeruli per kidney using computer image analysis by measuring the proportion of glomerular tuft comprised of extracellular matrix (ECM). The ratio of measured PAS-positive area (ECM) in the full glomerular area (GA) (i.e., ECM/GA) was used to indicate the magnitude of ECM accumulation as previously described (Song *et al.* 2004).

Immunohistochemistry

Serial sections at 4 μm in thickness were processed for immunohistochemical staining of proliferating cell nuclear antigen (PCNA), metallothionein (MT), and 3-nitrotyrosine (3-NT). Briefly, the sections were de-waxed in xylene and rehydrated through descending concentrations of ethanol and the endogenous peroxidases were blocked with 3 % H_2O_2/methanol for 15 min at 37°C. Sections were placed in a moist chamber and pre-incubated with a protein blocker solution containing 0.1 % BSA and 2 % non-immune goat serum to minimize non-specific staining. Sections were incubated for 1 h at 37 °C with the primary antibody diluted in an antibody diluting buffer (anti-PCNA antibody at 1/500 dilution, from DAKO, Carpinteria, CA, USA; anti-MT at 1/200 dilution and anti-3-NT at 1/200 dilution, both from Santa Cruz Biotechnology, Inc. CA, USA), and then with the biotinylated antibody (goat anti-rabbit IgG at 1: 300 dilution from DAKO, CA,USA) and the streptavidin-biotin-peroxidase complex (1:300 from DAKO) according to the manufacturer's recommendation. Peroxidases were observed with 3-3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma, Chicago, USA) at room temperature as the chromogen and the sections were briefly counterstained with hematoxylin. Negative controls were prepared by using 0.1 % PBS/BSA and non-immune rabbit serum instead of the primary antibody.

Slides were interpreted for antigen expression by two investigators without knowledge of the pathological information. Sections were considered as MT or 3-NT positive arbitrarily when more than 10% of cell nuclei and/or cytoplasm were stained for MT or 3-NT. MT or 3-NT negative sections showed no staining for MT or 3-NT. The intensity of MT or 3-NT expression was graded subjectively by two independent observers, and given a grade from 0 to 4, with a grade of 1 indicating no staining, and a grade of 4 indicating the greatest intensity of staining. A weighted score (HSCORE) was then generated to semi-quantify both MT and 3-NT expression levels in the tissue by multiplying MT or 3-NT intensity score with the proportion of positively stained renal tissues, based on a published method (McCarty *et al.*, 1985). The proliferation index (PI) was calculated as the number of PCNA positive cells in 100 cells in a field at × 200 magnifications.

Global DNA Methylation

Sections at 4 μm in thickness were placed in 0.01 mol/L citric acid (pH 6.0) in a microwave oven at full potency for 10 min. After the antigen retrieval, slides were immersed in 3.5N HCL for 15 min at room temperature to expose CpGs. The rest part of this staining is same as described above for other immunohistochemical staining. Sections were incubated with anti-5-methyl cytidine mouse monoclonal antibody (1/100; Abcam, Cambridge, MA, USA).

Statistical Analysis

Data are expressed as the mean ± SD. One-way ANOVA was used to determine if differences exist and if so, a post hoc Tukey's test was used for analysis of the difference between groups with Origin 7.5 laboratory data analysis and graphing software. Statistical significance was considered as $p < 0.05$.

RESULTS

Animal Model and Renal Metals

Previous exposure of rats to chronic Cd at very low level did not alter the body weight gain and animal survival rate (Data not shown). Relative weights of the kidney to body weight in Cd-treated rats were lower than that in control group only at the 20th week (Fig. 1B).

In control rats, renal Cd levels showed a slight increase in an age-dependent manner (Fig. 2A), while renal Zn, Cu and Ca did not significantly change as age increases (Fig. 2B,C, D). In Cd groups, renal Cd accumulation was significantly high at the 20th week and gradually decreased until the 52nd week where it remained high compared to control (Fig. 2A). In contrast to Cd concentration, renal Zn level was low in the Cd group at the 20th week ($P < 0.05$) and gradually returned to normal level at the 52nd week (Fig. 2B). Renal Cu was not significantly high in Cd groups from the 20th to 36th weeks, but significantly high in Cd groups from the 44th to the 52nd weeks, compared to controls (Fig. 2C). Renal Ca was slightly low in Cd groups from the 20th to 28th weeks and then gradually increased from the 44th week until 52nd week where it was significantly higher than that of the control (Fig. 2D).

Changes of Renal Function and Structure

There was no significant difference between controls and Cd groups for serum BUN and Cre levels at the most of time-points except for the time-point of 36th week where both serum BUN ($P < 0.05$) and Cre ($P < 0.01$) levels were transiently increased in the Cd group compared to the control (Fig. 3A, B).

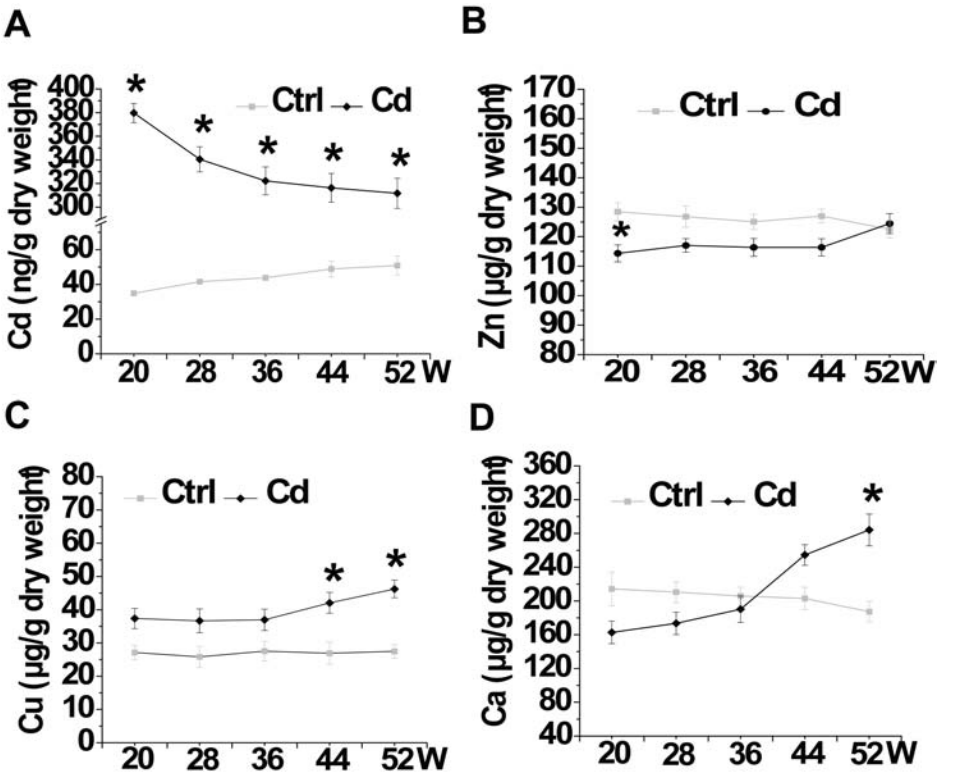

FIGURE 2. Renal metal contents. Renal tissues were weighted, dried and then measured with ICP-MS assay for Cd (A), Zn (B), Cu (C) and Ca (D) levels. Concentrations of these metals are expressed as ng or μg/g dry weight tissues. Data was presented as mean ± SD (n=6). $^{*}P < 0.05$ vs corresponding controls.

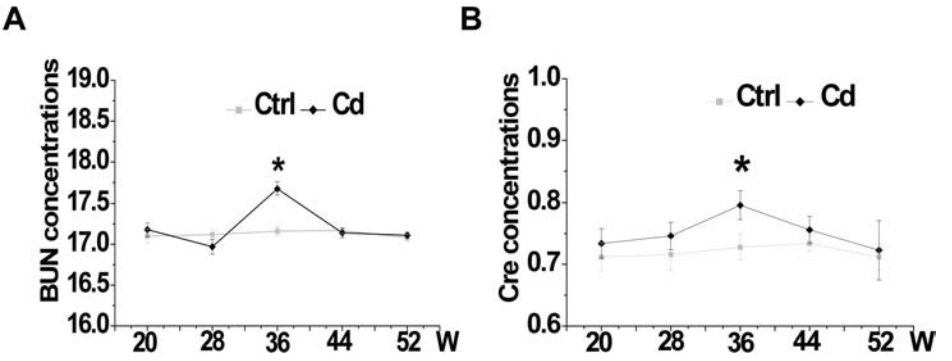

FIGURE 3. Changes of renal function and structure. Renal function was evaluated by BUN (A) and serum Cre (B) concentrations. Data was presented as mean ± SD (n=6). $^{*}P < 0.05$ vs corresponding controls. Histological changes of the kidney (C) were examined under light microscope with H&E staining at the indicated times. Arrows indicate the hyperplasia in the proximal tubule cells. Bar = 100 μm.

Histopathological analysis with H&E staining showed that renal tubules in controls were regularly arranged without damage. Renal epithelia in controls were dense, located in the basement of the lumen

and microvillus brush borders. In Cd groups, however, abnormal structure of renal tubules were present at the 20th week, with sparse lumens that varied in size and sparse epithelia that were physalides and dropsical and even moved upward to the lumen. Microvillus brush borders were wavy and some have shed, resulting in a shape of mesh. As the time increased, the above pathological changes in Cd groups were recovered gradually, but at the 44th week, hyperplasia, nuclear enlargement and chromatin thickening of individual cells in some renal tubules (see arrows) became evident and at the 52nd week, the hyperplasia cells were congregated at single tubule (Fig. 3C).

Renal fibrosis was examined with PAS staining (Fig. 4), which showed gradual increases in PAS density in renal tissues and a significant renal fibrosis, characterized by mesangial expansion and extracellular matrix accumulation of PAS-positive materials in Cd groups (Fig. 4A). Semi-quantitative analysis of mesangial index was performed based on PAS staining (Fig. 4B), using the criteria described in *Materials and Methods*. Previous exposure of rats to chronic Cd at very low level induced a significant increase in mesangial index.

Oxidative Damage and Antioxidant Levels in the Kidney

To examine the status of renal oxidative stress and damage, renal total glutathione (GSH) levels and superoxide dismutase (SOD) activities as antioxidant status were examined (Table 1). There was no significant change for GSH level at any time-point of the study. Renal SOD activity significantly decreased from the 36th week until the 52nd week of the study.

Renal oxidative damage was evaluated by renal tissue urea, lipid peroxidation (MDA) and protein nitration (3-NT). There was no significant difference between control and Cd groups for renal urea and MDA levels (Table 1). Immunochemical staining for 3-NT showed no positive staining in control groups (Fig. 5A), but a significant increase in staining intensity in Cd groups, which was strongest at the 20th week and then gradually weaken until the 52nd week where it remained high compared to control (Fig. 5A, B).

MT as both metal binding protein and antioxidant was also detected by immunochemical staining (Fig. 6). MT expressed in both glomeruli and tubules, mainly in the cell plasma (Fig. 6A). Semi-quantitative analysis of MT expression with the immunochemical score (HSCORE) system, as described in *Materials and Methods*, showed that MT levels in outer medulla were significantly higher in Cd groups with the highest level at the 20th week, and then gradually decreased until the 52nd week where it remained higher than that of control (Fig. 6B, C). The expression of MTs in proximal tubules of the inner medulla (staining images were not shown; semi-quantitative analysis shown in Fig. 6D) was similar to that of the outer medulla (Fig. 6C).

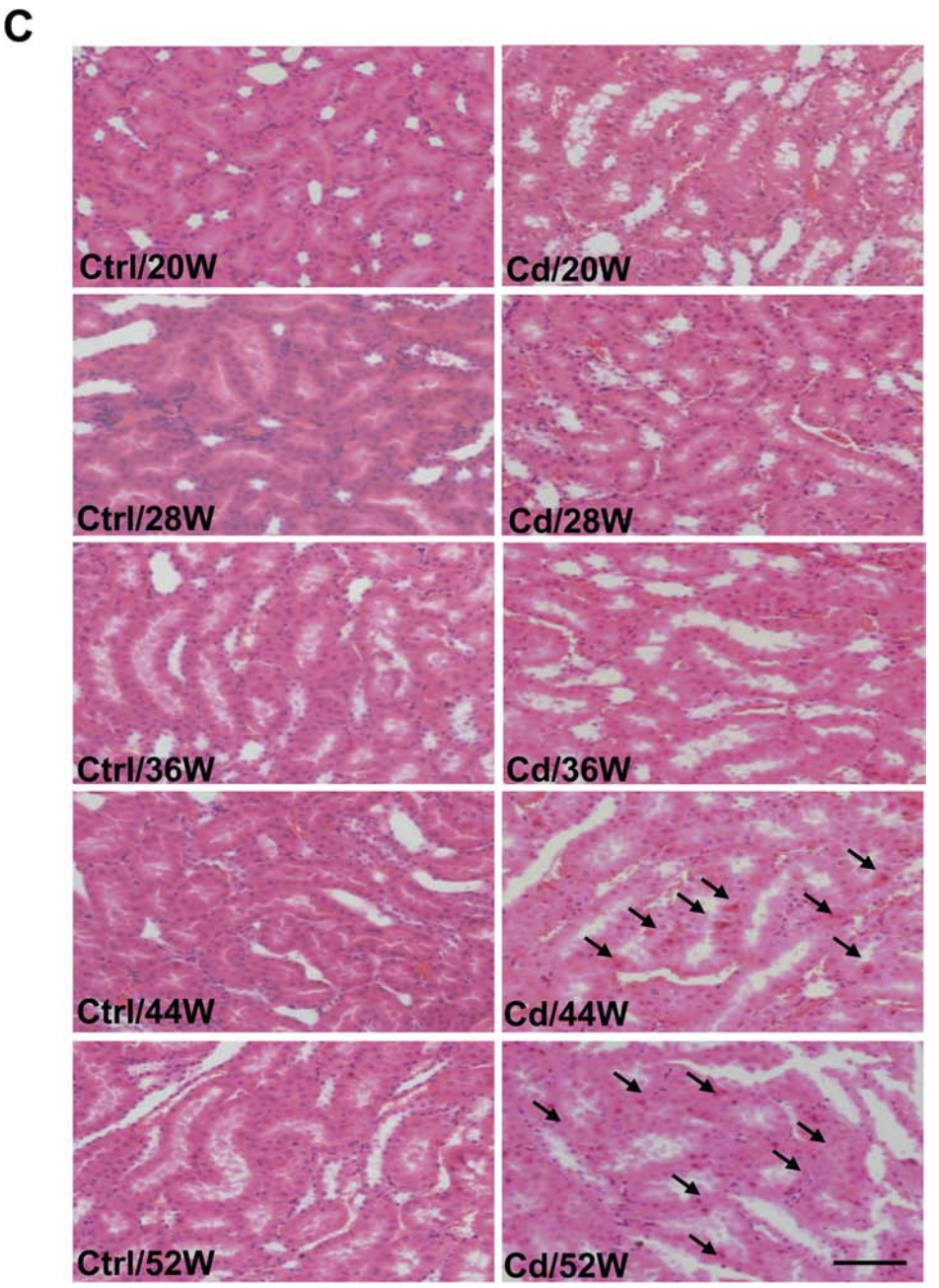

FIGURE 3. Continued

Renal Cell Proliferation

To evaluate whether chronic renal damage induced by Cd at very low level is accompanied with cell proliferation change, renal tissue was stained for PCNA. PCNA positive staining cells were observed in the kid-

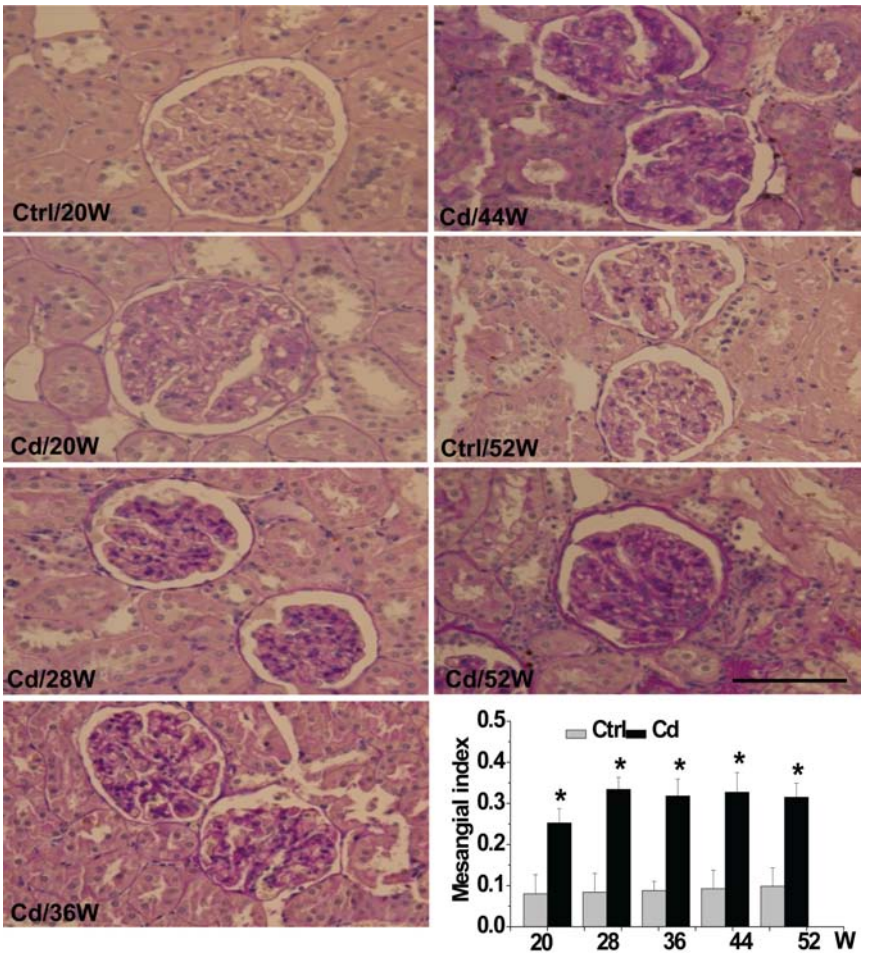

FIGURE 4. PAS staining for renal fibrosis. Renal sections from Cd-treated and control rats were stained for PAS, which showed increases PAS-positive materials in glomeruli. Bar = 100 μm. Renal histological changes were semi-quantitatively evaluated under light microscopy based on PAS staining for the mesangial damage index. Data was presented as mean ± SD (n=6). $^{*}P < 0.05$ vs corresponding controls.

TABLE 1. Data on Oxidative damage and antioxidant levels in the kidney tests at the indicated times.

Parameters	Group	20W	28W	36W	44W	52W
MDA (nmol/mg prt.)	Control	2.51±0.60	2.66±0.76	2.59±0.71	2.46±0.63	2.64±0.61
	Cd	2.44±0.66	2.63±0.76	2.64±0.73	2.53±0.41	2.57±0.58
SOD (U/mg prt.)	Control	49.02±2.43	52.21±2.58	51.05±3.80	53.92±3.17	53.12±1.51
	Cd	49.91±3.14	52.47±3.32	39.23±2.13*	43.85±1.86*	43.72±1.99*
GSH (gGSH/g prt.)	Control	15.05±0.86	15.78±0.84	15.19±0.45	15.95±1.07	15.32±1.28
	Cd	14.76±1.04	15.89±0.88	15.64±1.38	15.28±0.95	15.28±0.67
Urea (mg/dL)	Control	15.79±2.35	17.65±2.96	18.09±2.71	19.24±2.62	18.87±2.38
	Cd	16.19±2.72	17.02±1.99	17.91±1.58	19.68±1.09	19.02±2.89

Data were expressed as mean ± SD ($n = 6$); prt.: protein; $^{*}P < 0.05$ vs control.

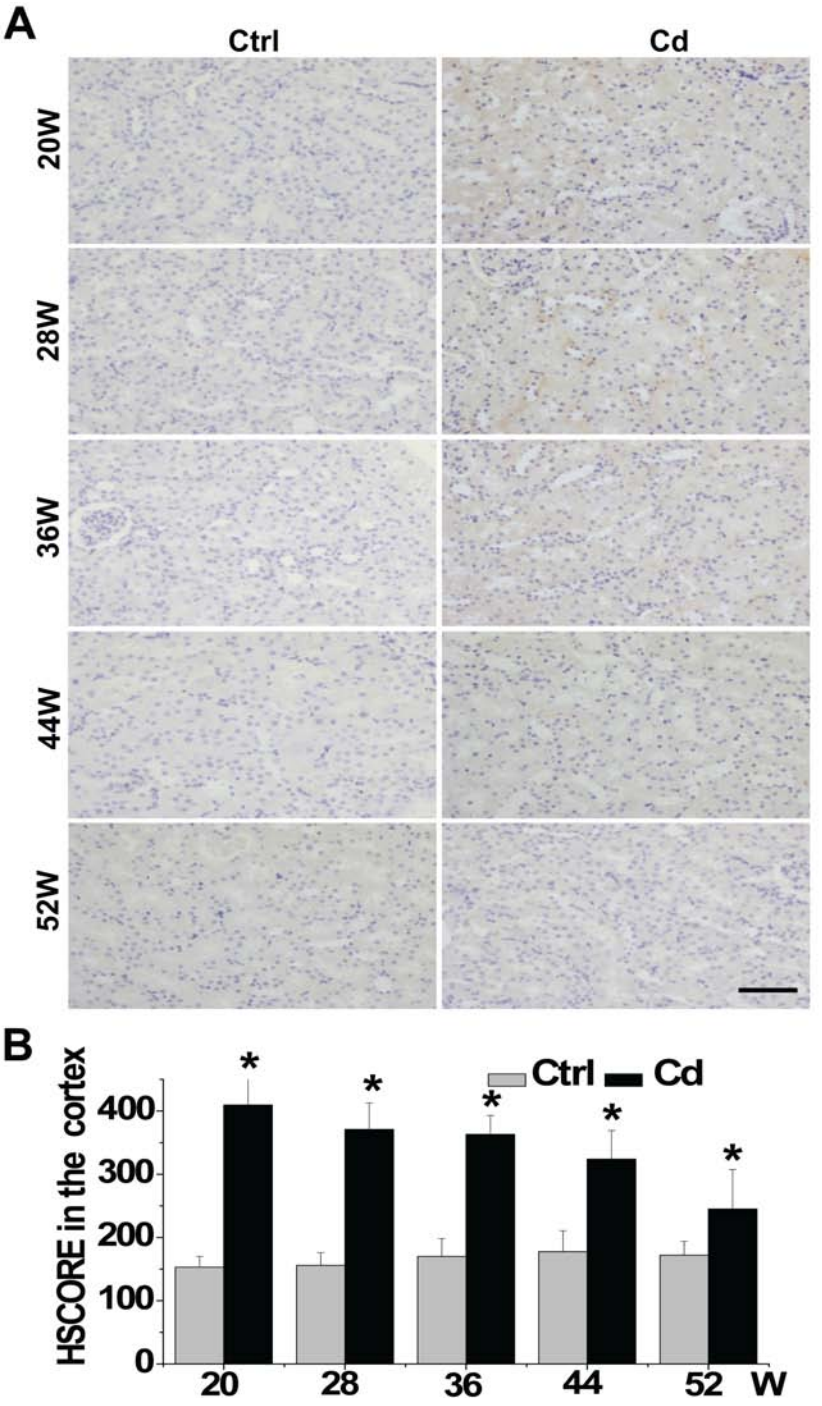

FIGURE 5. Immunohistochemical staining for 3-NT. (A) Representative microscopic images of the 3-NT, showing an increase in 3-NT accumulation in the kidney of Cd group compared to control. Bar = 100 μm. (B) Semi-quantitative analysis for the 3-NT accumulation in the renal cortex was performed with SCORE. Data was presented as mean ± SD (n=6). $^{*}P < 0.05$ vs corresponding controls.

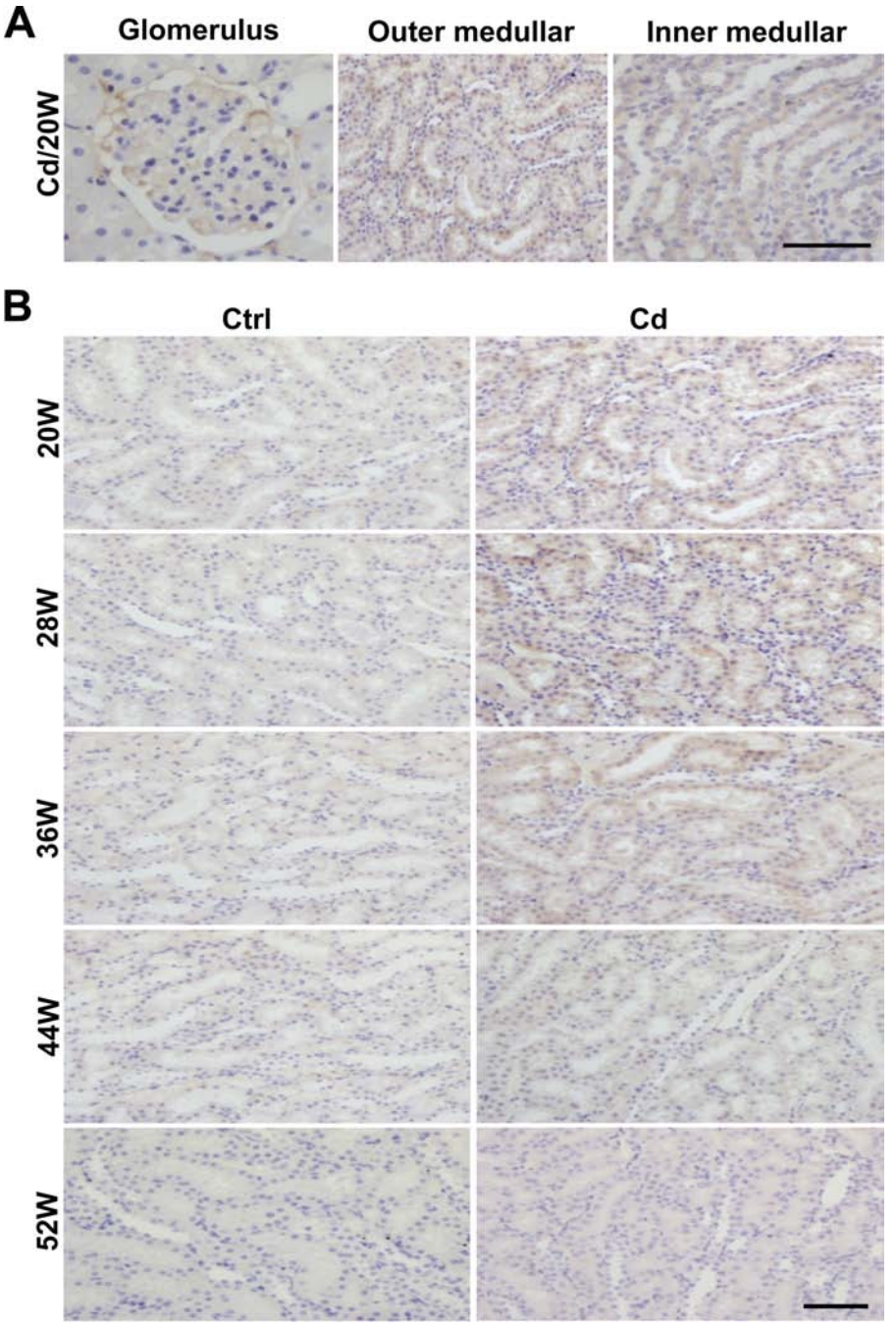

FIGURE 6. Renal MT expression by immunohistochemical method for MT. Renal sections were immunohistochemically stained for MT. A: Representative images of positive MT staining in three typical regions of the kidney. B: Representative images of MT staining in outer medullar from control and Cd groups. Bar = 100 μm. C, D: Semi-quantitative analysis for MT expression in the outer medullar and the inner medullar (staining images were not shown here), respectively, was performed with HSCORE. Data was presented as mean ± SD (n=6). $^{*}P < 0.05$ vs corresponding controls.

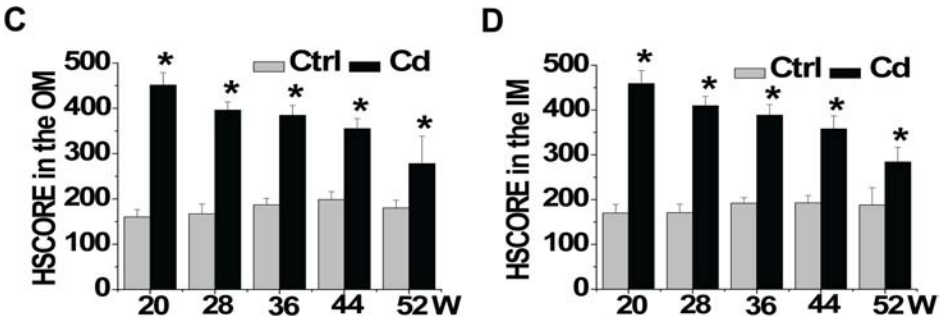

FIGURE 6. Continued

ney of Cd groups and were further evaluated separately by glomerular (Fig. 7A) and tubular (Fig. 7 B) parts. Mesangial cell proliferation was significantly lower at the 20th and 28th weeks and significantly higher at 52nd week in Cd groups than those in controls (Fig. 7A). Cell proliferation in the renal tubules was significantly lower at the 20th and 28th weeks, but significantly higher at the 44th and 52nd weeks in Cd groups than those in the controls (Fig. 7B). The finding of proliferative renal tubular epithelia cells (Fig. 7B) is consistent with the tubular hyperplasia at late time-points of the 44th and 52nd weeks shown in Fig. 3C.

Global DNA Methylation Status in the Kidney

Next the change of global DNA methylation status were examined for kidney tissues from controls and Cd groups by immunohistochemical staining with monoclonal antibody against 5-methyl-cytidine (Fig. 8). Cells with positive staining of DNA methylation were observed among glomerular and tubular epithelial cells in normal kidneys. In Cd groups, however, immunoreactivity of 5-methyl-cytidine in the kidney was markedly decreased at both early (the 20th week) and late (the 52nd week) time-points, compared to the controls.

DISCUSSION

The present study demonstrates, for the first time, the long-lasting effect of Cd on the kidney of the rats previously exposed to chronic Cd at very low level. We showed that previous exposure of rats to Cd at very low level did not affect body weight, but reduced the ratio of kidney/body weights at the early stage (likely before the 20th week). Renal Cd accumulation lasted for a long time even until 52 weeks (remaining about 5 times higher than that of control), although it gradually decreased from the 20th to the 52nd week. Renal Cd accumulation was correlated with slight decreases in renal Zn and Ca levels at the early stage (the 20th week), but not at late stages. Renal Cd accumulation was associated with

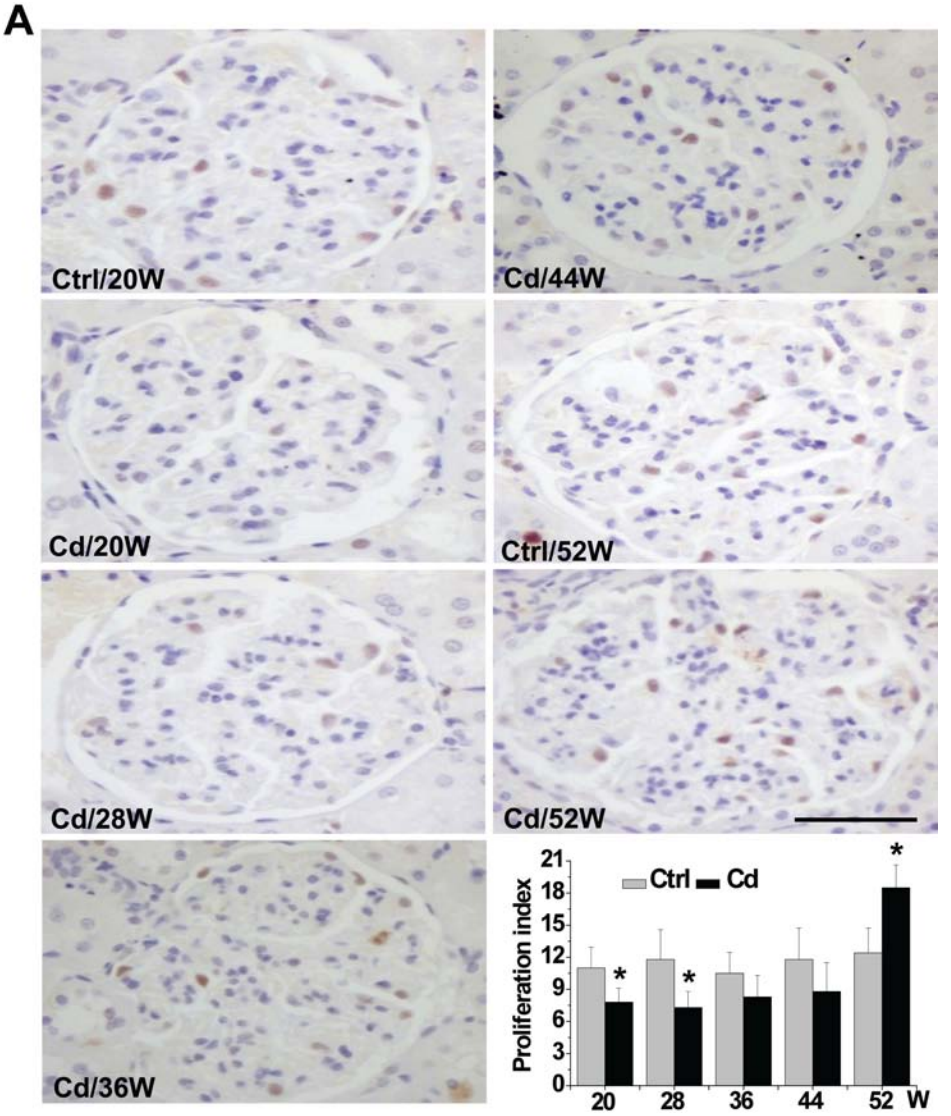

FIGURE 7. Immunohistochemical staining for proliferating cells. PCNA as an index of cell proliferation was examined by immunohistochemical stain in the glomeruli (A) and the renal tubular (B), followed by semi-quantitative analysis for the proliferation index (PI). Bar = 100 μm. Data was presented as mean ± SD (n=6). $^{*}P < 0.05$ vs corresponding controls.

renal nitrosative damage, measured by 3-NT accumulation, and increased MT expression. All these three measurements showed a time-dependent decrease from the 20th to the 52nd weeks of the study. Previous exposure to Cd at such low levels also induced the persistent renal fibrosis and cell proliferation. Immunohistochemical staining of 5-methyl-cytidine showed a persistent decrease in global DNA methylation.

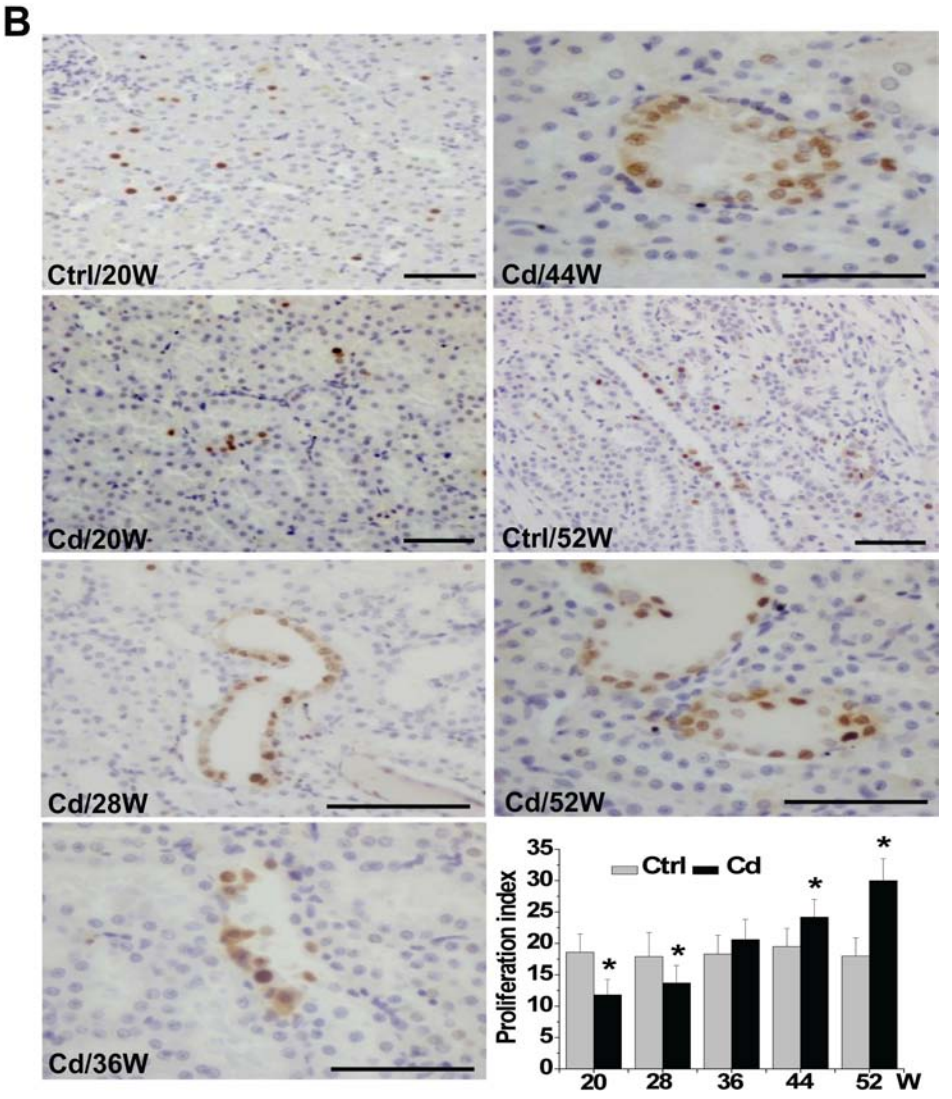

FIGURE 7. Continued

Renal accumulation of Cd in animals or humans exposed to chronic Cd has been extensively reported (Bernard 2004; Callegaro *et al.* 2010; Koizumi *et al.* 2008; Takaki *et al.* 2004; Zager 2005). Groten *et al.* (1991) have demonstrated that rats were fed with diets containing Cd as $CdCl_2$ either at 30 mg/kg that contributed a daily intake of Cd from 1-2.5 mg/kg body weight or at 1- 8 mg/kg that contributed a daily intake of Cd from 0.13 to 0.26 mg/kg body weight for 4 weeks. Both treatments resulted in a time-dependent increase in liver and renal Cd accumulation during the exposure period. Cd was predominantly distributed to the liver when rats were exposed to Cd at high doses while it was predominantly

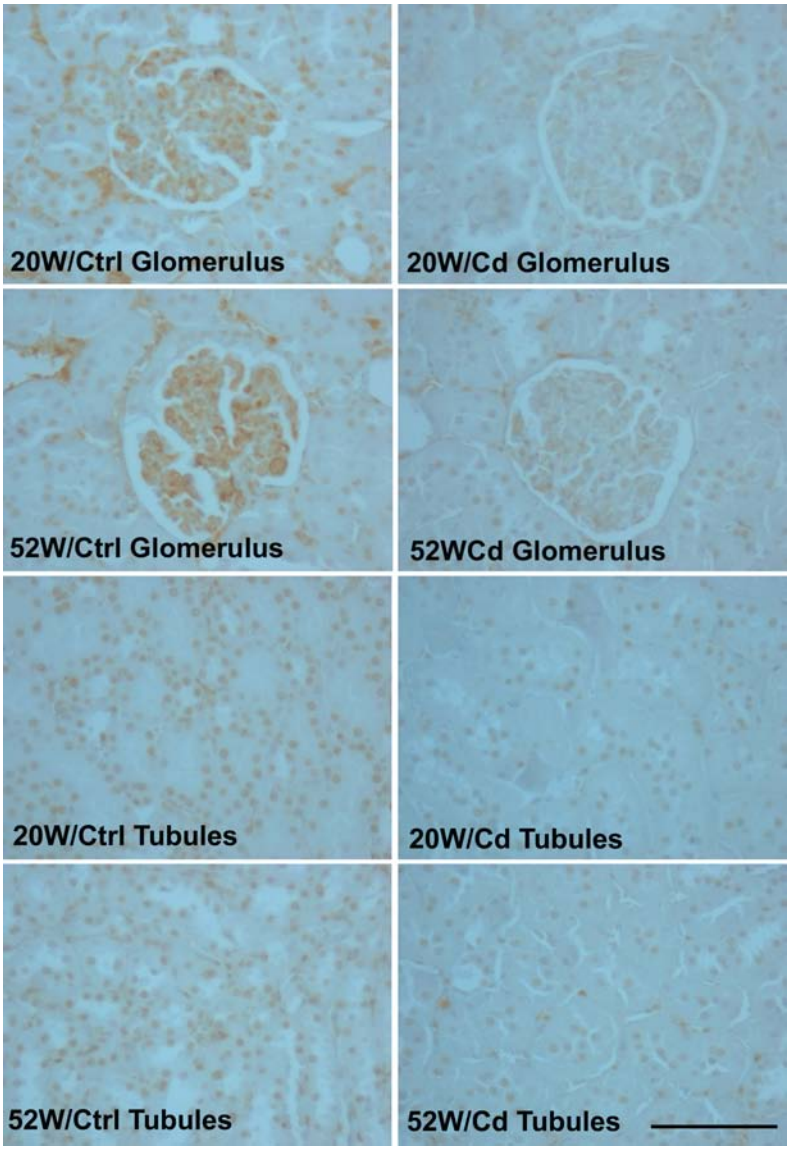

FIGURE 8. Immunohistochemical staining for global DNA methylation. Global DNA methylation status was examined with immunohistochemical staining for controls and Cd-treated kidneys, as described in *Materials and Methods*. Bar = 100 μm.

distributed to the kidney when rats were exposed to Cd at low doses. Up to date, however, whether previous exposure of animals or human to Cd at very low levels causes a long-lasting renal Cd accumulation remained unaddressed yet. Therefore, the first novelty of this study is to show that previous exposure of the rats to Cd at very low level resulted in a long-lasting renal Cd accumulation that, although gradually decreased, remained significantly higher than control levels even at the 52nd week (Fig. 2A).

The long-lasting accumulation of renal Cd is mainly due to the slow elimination of Cd as reported previously: approximately 0.001% of Cd in the body is excreted per day mostly via urine, and may be also due to redistribution from other organ's Cd such as the liver (Satarug and Moore 2004). The slow elimination of renal Cd accumulation is also reflected in control rats. From Fig. 1A, we found that there was a slight, but steady age- or time-dependent increase in renal Cd accumulation in the control group. The main reason may be the age-dependent accumulation of Cd intake from control diet that included very low levels of Cd from 0.1 mg Cd/kg non-dry weight (Asagba and Obi, 2005) or 1 mg/kg dry weight diet (Haouem *et al.* 2007). Haouem *et al.* (2007) have reported that when Wistar rats were fed with normal diet for 4 (16 weeks) to 12 months (48 weeks), they found the liver Cd accumulation from 20 to 40 ng/kg which is close to our finding for the renal Cd accumulation from 35 ng/kg at the 20th week to 50 ng/kg at the 52nd week of the study (Fig. 1A).

Studies have repeatedly indicated that Cd toxicity and carcinogenicity involve multiple steps: inducing reactive oxygen and/or nitrogen species (ROS and/or RNS) generation, depressing antioxidant function, and inhibiting the enzymes responsible for DNA repair (Hradilova *et al.* 2010; Schwerdtle *et al.* 2010; Waalkes 2000). Tissues exposed to acute or chronic Cd at relative high doses often showed the oxidative damage, mirrored by increased renal MDA contents and decreased SOD activity during or at the end of Cd treatment (Amara *et al.* 2006; Renugadevi and Prabu 2010; Xu *et al.* 2010). However, no study has attempted to define whether these oxidative endpoints persistently exist if examined at very late stage after Cd treatment. In the present study, we did not observe significant increases in oxidative damage, measured by renal tissue urea and MDA levels at the 20th to 52nd weeks of the study (Table 1). Since we did not measure the MDA contents during Cd treatment, it is unclear whether renal lipid peroxidation increased during Cd-treatment, which may become a limitation for the present study.

Regarding the fact that although there was a long-lasting renal Cd accumulation, there was lack of a long-lasting oxidative renal damage, we need to consider the following reasons: (1) Age may be one of the susceptible determinates to Cd-induced renal lipid peroxidation. Shibasaki *et al.* (1996) have reported that when they treated both young and old hamsters with Cd, they found that renal MDA content was significantly increased only in young animals exposed to Cd. In the present study, the rats are relative old: i.g. about 26 weeks old at the earliest time-point (6 week old before starting study + 20 weeks of the study) and 58 weeks old at the latest time-point (6 weeks + 52 weeks); (2) It was reported that renal MDA was found to be increased in response to acute Cd exposure, and did not increase or even decrease in response to chronic Cd exposure (El-Maraghy *et al.* 2001); (3) MDA measurement may not sensitive

enough to detect the mild increase in MDA level in whole kidney; therefore, immunohistochemical staining of 3-NT was also used as an index of protein nitration (a nitrosative damage) in the present study. We showed a significant increase in 3-NT accumulation predominantly distributed in renal tubules at the 20th week and gradual decrease until the 52nd week (Fig. 5). Several key antioxidants in tissue cells, most notably SOD and catalase, were reported to be decreased in response to Cd treatment (Uchida *et al.* 2004; Waisberg *et al.* 2003). We also found that the total SOD activity decreased, starting from the 36th week (Table 1). This suggests that Cd may not produce significant free radical species by itself, but rather impair the normal free-radical scavenging system (Stohs *et al.* 2001). Regarding to the mechanism responsible for the effect of Cd in decreasing renal SOD activity, we know that most of the antioxidant enzymes become inactive by Cd exposure due to the direct binding of Cd to enzyme active sites if they contains SH groups (Quig 1998) or displacement of metal cofactors from active sites (Casalino *et al.* 2000). For instance, Bauer *et al.* (1980) have reported that Cd^{111} was able to occupy the site of Zn in the CuZn–SOD molecule creating an inactive form of the enzyme ($CuCd^{111}SOD$).

MT is a cysteine-rich low molecular weight protein, which can bind excess heavy metal ions preventing their toxic effects, and also sequesters several ROSs and RNSs to play an important role in protecting against oxidative stress (Cai *et al.* 1999; Cai *et al.* 2000; Cherian *et al.* 2003; Sato and Bremner 1993). Cd is readily able to induce MT expression in the liver and kidney (Cherian *et al.* 2003). We found that MT expression was up-regulated in both tubules and glomeruli of the kidney at the 20th week and then was gradually decreased in a time-dependent manner until the 52nd week, but remained higher than control level (Fig. 6). Up-regulated renal MT expression may be induced by the accumulated Cd in the kidney so that MT may be a protective mechanisms at the early stage to prevent the kidney (Sato and Kondoh 2002).

In addition, MT up-regulation in the kidney may be responsible for the long-lasting Cd accumulation. MT binds Cd with a high affinity, resulting in a decreased availability of free Cd^{2+} capable of interacting with cellular targets to avoid the acute toxicity of Cd. These assumptions are supported by the fact that mice with lack of MT genes are highly susceptible to Cd-induced renal toxicity (Liu *et al.* 1998; Liu *et al.* 2000). However, although renal MT binds Cd that avoids acute renal damage, MT that bound Cd in the kidney may remain slowly releasing Cd that continually induces mild renal damage. Our results showed persistent pathological lesions in the kidney, including tubular degeneration, glomerular cell proliferation and swelling, and interstitial fibrosis. Other studies also showed that exposure to Cd at low levels can cause renal damage (Jarup *et al.* 2000).

In the present study, we used immunohistochemical detection of PCNA. We found a significant increase in renal glomerular and tubular cell proliferation at the late stage (Fig. 7). PCNA is a marker for cells in early G1 and S phases of the cell cycle. Chronic exposure to Cd can damage renal proximal tubular epithelial cells of S1 and S2 segments (Conner and Fowler 1993). Our results indicate that the previous exposure of rats to chronic Cd at very low level interfered with the normal synthesis of DNA, including the inhibitive effect on cell proliferation at early stage and stimulating effect on DNA synthesis and cell proliferation at the late stage (Fig. 7). In our recent study, we observed a persistent increase of hepatic cell proliferation in the rats at 44th and 52nd weeks after chronic exposure to Cd at low dose (Wang *et al.* 2011).

Many studies over the years have yielded evidence that Cd may contribute to renal cancers in humans (Waalkes 2003). Our study showed that the structure of renal tubules was impaired at the early phase and proliferative cells were observed in the later phase, which could be a pathological basis of renal carcinogenesis. Currently Cd is predominantly considered as a non-genotoxic carcinogen (Jiang *et al.* 2008). However, Cd is known to bind to specific sites in chromatin, and such binding might possibly interfere with the transcription of certain genes. Epigenetics can be described as a stable alteration in gene expression without any change in gene sequence (Klein 2005). Reportedly Cd is an effective inhibitor of DNMTs and induces DNA hypomethylation *in vitro* (Takahashi *et al.* 2003). Consistent with this notion, we demonstrated that global DNA methylation was markedly diminished in the glomeruli and tubules of kidneys from Cd-treated rats at both early (the 20th week) and late (the 52nd week) stages (Fig 8). These results imply that previous Cd exposure even at very low level may disrupt DNA methylation, as previously observed *in vitro* studies (Jiang *et al.* 2008; Singh *et al.* 2009), which may explain the long-lasting effect of Cd on renal cell proliferation and may also be one of the possible underlying carcinogenic mechanisms of Cd. However, we do not have any further evidence now to determine whether there will be a real risk for the development of renal cancers in these Cd-treated rats under the present experimental conditions, and if so, whether it will be really related to the aberrant methylation.

In summary, we have studied renal functional, structural and biochemical changes in the rats previously exposed to chronic Cd at very low level. We demonstrated the persistent damaging effect on the kidney of these rats. The renal accumulation of Cd can last at high level for 52 weeks, which was accompanied with mild oxidative damage, and persistent cell proliferation and DNA hypomethylation. Whether these pathogenic alterations are causative of the development of renal dysfunction or possible cancer remains further exploration. In addition, whether there will be gender-dependent effects remains unclear since we did not

include female in the present study, which will be also warranted in the future studies.

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