Effects of paternal cadmium exposure on the sperm quality of male rats and the neurobehavioral system of their offspring

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Abstract. Cadmium (Cd) is a testicular toxicant and an endocrine disruptor in humans and rodents. The aim of the present study was to investigate the effects of paternal Cd exposure on the sperm quality of male rats and the neurobehavioral system of their offspring. A total of 12 male rats were randomized into a control and Cd-treated group (n=6 per group), and 12 female rats were administered distilled water and randomly divided into two groups (n=6 per group). Subsequently, sperm motility, viability, malformation rate of male rats and the neuromotor maturation, antioxidant ability, Cd accumulation in different organs of their offspring were measured. Compared with the control rats, the sperm motility rate and vitality were significantly reduced (P<0.01) and the sperm malformation rate was significantly increased (P<0.01) in the male rats following Cd treatment. Regarding the nervous system development of the offspring, the cliff-avoidance reflex, surface-righting reflex and negative geotaxis results exhibited significant differences between the Cd exposure and control groups (P<0.05). The Cd content in the liver and heart of the offspring of the Cd exposure rats was higher than that in the control rats (P<0.05), and the liver content peaked on postnatal day 21. Furthermore, Cd exposure affected the antioxidant activity of the offspring, which was shown by glutathione, malondialdehyde and superoxide dismutase assays. Collectively, the results indicate that Cd exposure affects the sperm quality of male rats and the neurobehavioral system of their offspring.

Introduction

Cadmium (Cd) is a heavy metal and one of the major environmental toxicants. The general population is exposed to Cd by ingestion or inhalation (1). The Cd content in the air is ~0.04 μg/m^3; in drinking water, it is <1 μg/l, which is not of particular concern. Much of the Cd entering the body comes from terrestrial foods (2,3). For the average individual, 1- 3 μg Cd is absorbed via food every day (4). In addition, Cd, having a long biological half-life in humans, particularly accumulates in the kidneys and liver (1). More significantly, Cd has been identified as a severely adverse element for the mammalian reproductive system and causes considerable damage to the ovaries and testes (5). Consequently, it can affect the offspring to a certain extent.

Cd can increase lipid peroxidation through the generation of noxious radicals, such as superoxide anion radicals, hydroxyl radicals, nitric oxide and hydrogen peroxide (6). Findings from numerous studies have confirmed the proposal that oxidative stress can play a significant role in the etiology of defective sperm formation, function and count profile, as well as male infertility (7-9). Germ cells are more subject to oxidative stress than somatic cells, thus contributing to the alteration of enzyme activities and various important signal transduction pathways and, in turn, affecting fertility (10,11).

The effects of maternal Cd exposure on offspring have been well-documented (12-14). Maternal Cd exposure during pregnancy was shown to markedly reduce serum testosterone levels, while the placenta could deter most of the Cd from passing from dams to fetuses (13); however, few studies that have investigated the effects of paternal Cd exposure on offspring are rare. Consequently, the aim of the present study was to investigate whether paternal Cd exposure could affect the offspring and to explore how any effects were expressed. Since humans are chronically exposed to Cd via ingestion or absorption from food and drinking water, the gavage method was adopted in this study to simulate the real Cd exposure pathway, and the effects of paternal Cd exposure on the sperm quality of male rats and the neurobehavioral system development of their offspring were investigated.

Materials and methods

Chemicals. Cadmium chloride (CdCl$_2$), methanol, sodium hydrogen phosphate and sodium dihydrogen phosphate were of analytical grade and were purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). Considering the quantitative exposure and tissue retention capacity of humans, a dose of 22.15 mg/kg body weight was selected for the study.

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Animals and treatments. Sprague Dawley rats (5-6 weeks old; male rats, 180-220 g; female rats, 60-80 g) were obtained from Weitonglihua Experimental Animal Technical Co., Ltd. (Beijing, China). Rats were housed in cages and acclimated to laboratory conditions (temperature, 23±2°C; 12-h light/dark cycle; humidity, 50±5%) and fed a balanced diet and water *ad libitum* for 1 week prior to the experiments. Twelve male rats were selected and randomly divided into a control and a Cd-treated group (n=6/group). In addition, 12 female rats were administered distilled water and randomly divided into two groups (n=6/group). The control and Cd-treated groups of male rats were gavaged distilled water and $CdCl_2$, respectively, every 2 days for 9 weeks in total. For mating purposes, the control group was housed in the same cage as one group of female rats for 1 week, while the Cd-treated group was housed with the remaining female rats. During the mating process, measures were taken to avoid the cross-contamination of Cd between male and female rats. The presence of a vaginal plug was designated as gestational day (GD) 0. After GD 0, the male rats were separated from the female rats. All experimental protocols and procedures were approved by the Animal Ethics Committee of China Agricultural University (R2012072; Beijing, China).

Sperm motility, viability and malformation rate. Sperm motility and viability were detected using an automatic semen analyzer (Songjing Tianlun Biological Science and Technology, Co., Ltd., Nanning, China). The spermatozoa were classified as motile or immotile. Sperm motility was expressed as the percentage of motile sperm. Sperm viability was assessed via eosin-nigrosin staining. Unstained, live sperm were differentiated from pink-stained, dead sperm, and their numbers were calculated. A 50-μl aliquot of sperm suspension was diluted 20 times in phosphate-buffered saline at 37°C, smeared gently on a glass slide with methanol and dyed using 1% eosin Y for 1 h. The number of malformed sperm was recorded under a light microscope at high magnification, and the sperm malformation rate was calculated. Malformed sperm included an abnormality of the head, rump and body.

Neuromotor maturation assessment of the offspring
Righting reflex. In this study, the righting reflex included the surface-righting reflex and the air-righting reflex. The surface-righting reflex was evaluated on postnatal days (PNDs) 3-9. The latency time for a pup placed on its back to turn over and place all four paws on the floor was recorded. The air-righting reflex was measured on PNDs 10-15. A pup was placed in the air on its back and dropped 25 cm to a pad on the floor. Success was defined by the four paws touching the floor smoothly, and the number of successful trials was recorded. Every pup was tested with three consecutive trials. A maximum time of 30 sec was allowed.

Cliff avoidance. Cliff avoidance was evaluated on PNDs 4, 5, 7 and 9. Pups were placed with the forepaws and face on the edge of a table top. The latency to return the body 1.5 cm from the 'cliff' was recorded. Subjects were given a maximum time of 30 sec per trial.

Negative geotaxis. The negative geotaxis reflex test was conducted every other day between PNDs 2 and 10. A pup was oriented toward the top when placed in a head-down position
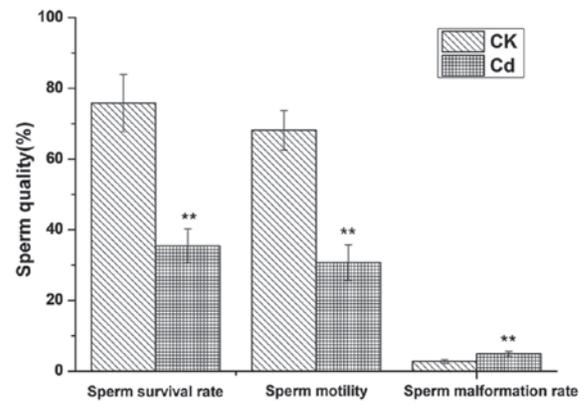

Figure 1. Effect of Cd exposure on sperm quality. A 50-μl aliquot of sperm suspension was diluted 20 times in phosphate-buffered saline at 37°C, smeared gently on a glass slide with methanol and dyed using 1% eosin Y for 1 h. **P<0.01 vs. CK. Cd, cadmium; CK, control group.

on a board inclined at 25°, and the latency to rotate 180° was measured. The trail was considered to be a success when the pup turned 180° within the allotted time of 3 min. The number of successful trials and the time taken to turn the 180° were recorded.

Forelimb grip strength. On PNDs 10-15, forelimb grip strength was measured. Each pup was suspended by its forefeet from a fixed wire, and the duration that each pup held on to the wire was recorded.

Cd accumulation in different organs. The liver, kidney, heart, brain and left testis of the newborns were incinerated according to the method described by Wickliff *et al* (15). Cd accumulation was determined by inductively coupled plasma atomic emission spectrometry (Varian Medical Systems, Inc., Palo Alto, CA, USA) with a detection limit of 15 ng/ml for blood and 30 ng/g for solid samples.

Biochemical assays. The glutathione (GSH), superoxide dismutase (T-SOD) and malondialdehyde (MDA) levels of the tissues (liver, brain, heart, kidney and testis) of the newborns were determined. According to the method described by Kuo *et al* (16), GSH was measured in deproteinized supernatant fractions from 10% tissue homogenates, using 0.04% 5,5'-dithiobis-(2-nitrobenzoic acid) in 10% sodium citrate (Sigma-Aldrich, St. Louis, MO, USA). The absorption at 412 nm was recorded using a spectrophotometer (Agilent Technologies, Inc., Palo Alto, CA, USA). SOD in the tissues was determined according to the method of Kakkar *et al* (17). Lipid peroxidation was measured by the MDA formed. The 10% tissue homogenate was mixed with 150 mM KCl for 30 min at 37°C, and the MDA was then determined using the thiobarbituric acid reaction (Sigma-Aldrich) (18).

Statistical analysis. Results are presented as the mean ± standard error. The data were statistically analyzed using one-way analysis of variance followed by Duncan's test. The statistical analysis was conducted using SPSS software, version 11.5 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.
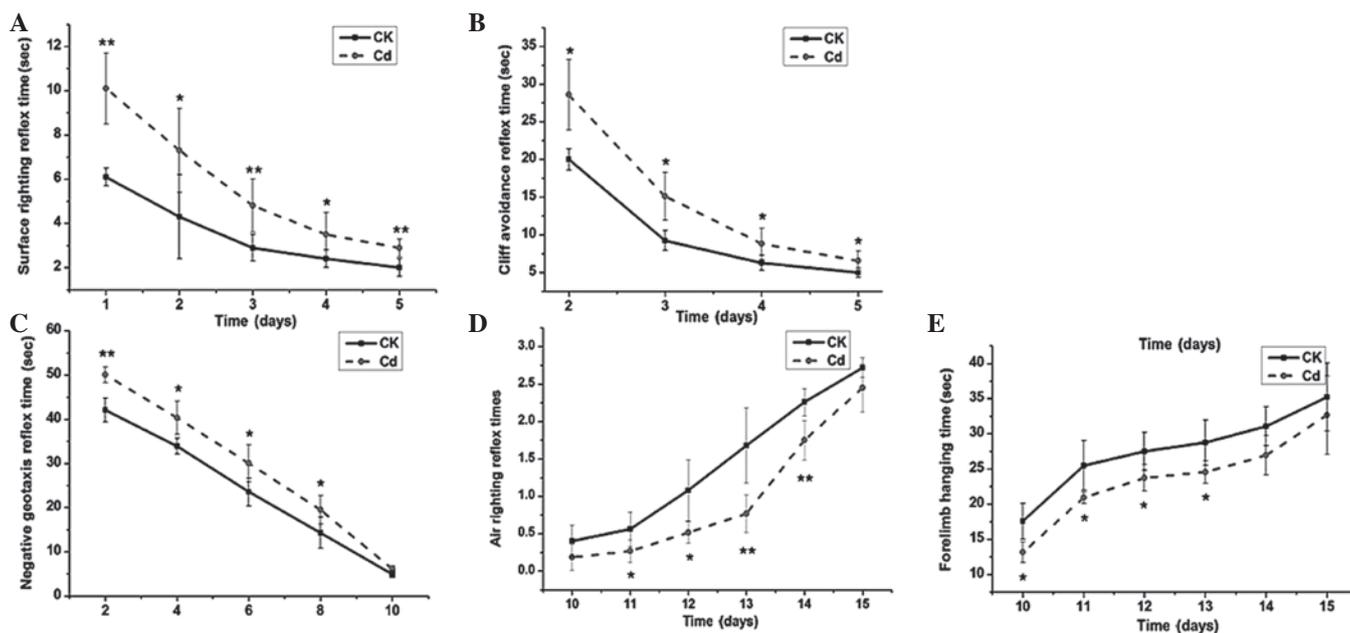
Figure 2. Effect of paternal Cd exposure on the (A) surface-righting reflex, (B) cliff avoidance, (C) negative geotaxis, (D) air-righting reflex and (E) forelimb grip strength of rat pups. *P<0.05 and **P<0.01. Cd, cadmium; CK, control group.
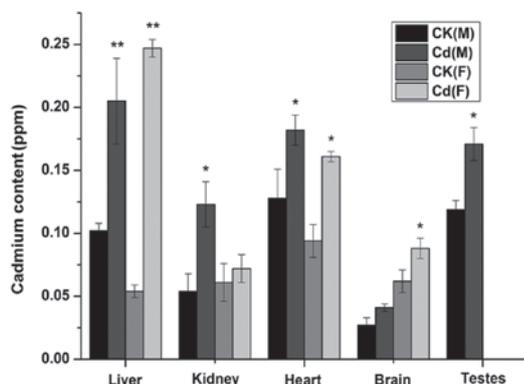

Figure 3. Cd content in different organs of rat pups on postnatal day 21. *P<0.05 and **P<0.01 vs. the corresponding CK. Cd, cadmium; CK, control group; M, males; F, females.

Results

Effect of Cd exposure on sperm motility, viability and malformation rate. Fig. 1 shows the effect of Cd exposure on epididymal sperm motility and viability, as well as the malformation rate. The Cd-treated group showed significantly lower sperm motility and viability when compared with the control group (P<0.01). In addition, the sperm malformation rate of the Cd-treated group was significantly higher than that of the control group (P<0.01). The abnormalities of the malformed sperm included large heads, decollation, and folding and broken tails. The sperm quality of the adult male rats decreased markedly following Cd exposure.

Effect of Cd exposure on neuromotor maturation of the offspring. The neuromotor maturation of the offspring of the Cd-treated group, assessed via surface- and air-righting, cliff avoidance and negative geotaxis reflexes, as well as forelimb grip strength, was

found to be significantly suppressed throughout the test period (Fig. 2). Compared with the control group, the surface-righting (Fig. 2A), cliff avoidance (Fig. 2B) and negative geotaxis (Fig. 2C) reflex times of the Cd-treated group were significantly longer than those of the control group (P<0.05), while the number of successful air-righting reflex trials (Fig. 2D) and the forelimb hanging times (Fig. 2E) of the Cd-treated group were significantly lower than those of the control group (P<0.05).

Cd accumulation in different organs of the offspring. For the offspring on PND 21, the Cd levels in the liver and heart of male and female newborns in the Cd-treated group were significantly higher than those of the control group (liver, P<0.01; heart, P<0.05) (Fig. 3). Furthermore, the Cd content in the kidney was significantly higher in the Cd-treated male newborns than that in the control male newborns (P<0.05); however, the difference between the Cd-treated female newborns and control female newborns was not significant (P>0.05). The opposite phenomenon was exhibited in the brain. In addition, the testis Cd concentration was significantly lower in the male newborns of the Cd-treated group than that in the control group (P<0.05). The data for the Cd-treated group could be summarized as follows: Cd accumulation was highest in the liver for male and female newborns; Cd accumulation was lowest in the brain and kidney for male and female newborns, respectively.

Biochemical assays. At PND 21, no significant differences were found between the Cd-treated and control groups in terms of the T-SOD levels in the liver, kidney, heart and brain of the male and female newborns (P>0.05); however, the difference in the testes was statistically significant (P<0.01). At PND 70, however, the T-SOD levels in all tissues of the Cd-treated male and female offspring were significantly decreased compared with those of the control group (P<0.05) (Fig. 4A). As shown in Fig. 4B, at PNDs 21 and 70, the GSH levels in all tissues of
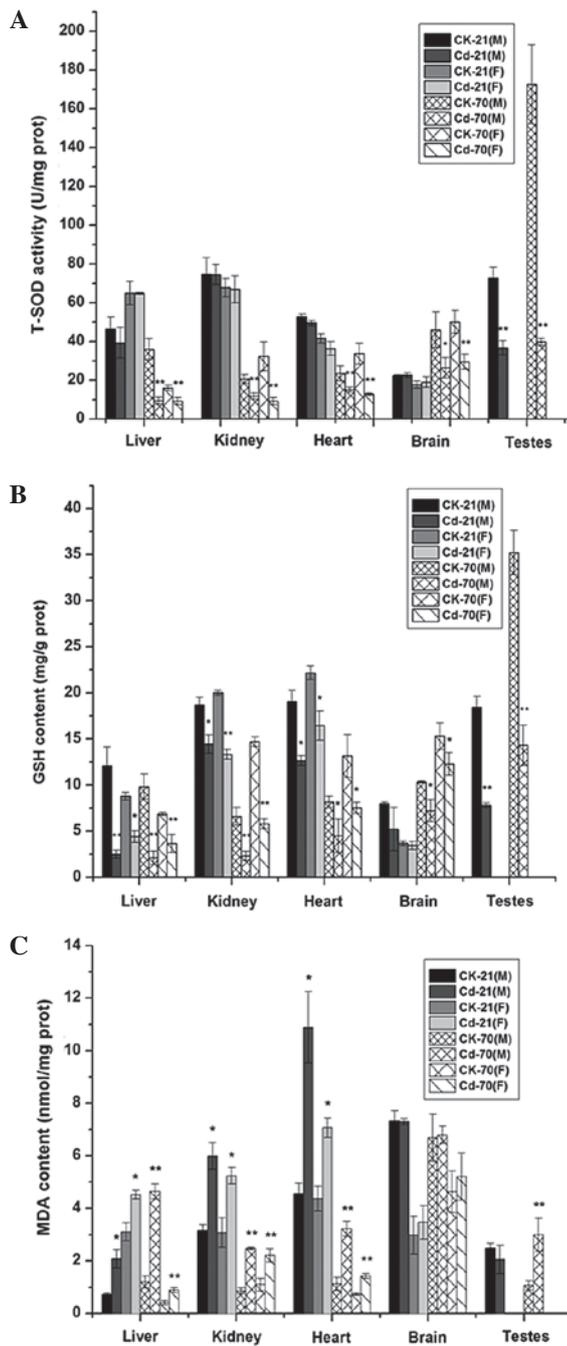
Figure 4. Effect of paternal Cd exposure on the (A) T-SOD activity (B) GSH content and (C) MDA content at postnatal days 21 and 70. *P<0.05 and **P<0.01 vs. the corresponding CK. Cd, cadmium; CK, control group M, males; F, females; T-SOD, superoxide dismutase; GSH, glutathione; MDA, malondialdehyde.

MDA content in all tissues of the Cd-treated group, with the exception of the brain, was significantly increased compared with that of the control group.

Discussion

The present study showed that, for adult male rats, sperm quality was significantly affected by the administration of CdCl$_2$. Sperm motility, which is mainly a post-testicular factor, is vulnerable to reproductive toxicants (19,20) and relies on a maturation process in the epididymis. When the rats were exposed to Cd, it is likely that the synthesis of epididymal proteins and other substances associated with sperm maturation was affected and structural and biochemical changes in the spermatozoa were produced. Thus, Cd exposure contributed to a decrease in sperm motility and an increase in the sperm malformation rate.

For the newborns of the Cd-treated group at PND 21, the Cd concentration in the liver, kidney, brain, testes and heart was significantly higher than that observed in the control group tissues, particularly in the liver. Neonate rats exhibit significantly increased Cd absorption and storage compared with adult rats (21). The observed result was therefore likely due to paternal Cd exposure affecting the ability of the offspring to absorb and store Cd; however, the specific mechanism is unclear.

Cd exposure can generate neurobehavioral disturbances, including reductions in attention, psychomotor speed and memory (22). It has been suggested that Cd is more toxic in newborn than in adult rats due to its ability to diffuse across all biological membranes, thus allowing Cd penetration through the blood-brain barrier (BBB) (23). The Cd administration initially affects the integrity and permeability of the vascular endothelium, and the necrotic changes in nerve cells are only secondary to this effect, which results in edema and interference with oxygen and nutrient uptake into the brain (24,25). In the present study, significant suppression of all reflexes, including surface righting, air righting, cliff avoidance, forelimb grip strength and negative geotaxis, clearly suggested a direct effect of Cd on the neuromotor maturation of the pups.

The effect of Cd has been demonstrated by increased lipid peroxidation and enzyme inhibition (26). Treatment with Cd can induce decreased T-SOD activity and GSH content and an increase in the MDA content, which is consistent with the Cd burden of different tissues (27). These changes appear to be due to the generation of reactive oxygen species (ROS) (28). GSH is a major component of the oxidant defense system, which acts to scavenge free radicals generated during Cd intoxication. It has been well-documented that reactive intermediates can react with GSH and undergo transformation into oxidized GSH, either through a direct chemical reaction or through a glutathione transferase-mediated reaction (29). In the present study, the Cd-treated group at PNDs 21 and 70 exhibited significant changes in the T-SOD activity and GSH and MDA levels of the tested tissues, with the exception of the brain; these findings most likely resulted from the difference in the Cd absorption and storage abilities between different tissues.

It is believed that brain tissue is particularly sensitive to oxidative stress. Neurons are abundant in mitochondria and have a highly aerobic metabolism system; nearly 20% of total oxygen may be used by the brain. During mitochondrial respiration, ≤2% of the consumed oxygen may be converted

the Cd-treated newborns, with the exception of the brain at PND 21, were significantly depleted compared with those of the newborns of the control group. The results of the MDA content are shown in Fig. 4C. At PND 21, significant differences were found between the offspring of the Cd-treated group and the control group in terms of the MDA content of the liver, kidney and heart in both males and females (P<0.05); however, the brain and testis MDA levels in the offspring of the Cd-treated group were not significantly higher than those of the control group offspring (P>0.05). For the offspring at PND 70, the

to ROS, and this proportion increases with oxygen consumption (30). Thus, ROS content in the brain may be higher than that in any other organ. In addition, due to the increase in Cd content in the brain of newborns, the oxidant system is damaged, as demonstrated by a decrease in T-SOD activity and GSH content. Thus, the above effects may combine to make the brain a preferential target for oxidative stress-related degeneration (31). It has been reported that numerous neurodegenerative illnesses, such as Parkinson's and Alzheimer's disease and amyotrophic lateral sclerosis, result from the increment of ROS and oxidative stress (32). Furthermore, it has been found that iron accumulation in the brain induces neurodegenerative disorders via oxidative stress mechanisms (33). It is possible that Cd accumulation is closely associated with neurobehavioral function.

In the present study, it was concluded that Cd exposure in adult male rats could significantly affect the sperm quality. In addition, paternal Cd exposure could exert significant effects on the neurobehavioral system of the offspring. These findings may be explained by the fact that Cd is more toxic in newborn than in adult rats due to its ability to diffuse across all biological membranes, thus allowing Cd penetration through the BBB. Furthermore, Cd accumulation in the brain can damage the oxidative system, decreasing T-SOD activity and GSH content, which results in neurodegenerative disorders or the damage of the neurobehavioral system.

Acknowledgements

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