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RESEARCH ARTICLE

Hypomethylation of LINE-1 retrotransposons is associated with cadmium-induced testicular injury

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Abstract

Retrotransposons, as vital regulator of male fertility, are essential for spermatogenesis. Cadmium (Cd) is an environmental toxicant and endocrine disruptor, targeting the reproductive system. Growing evidence shows that Cd exposure can induce male infertility in mammals. In this study, we generated a male C57BL/6 J mice model with consecutive 35 days cadmium chloride ($CdCl_2$) in different concentrations of 0, 0.25, 0.5, 1.0, and 2.0 mg/kg. The results indicated that 1.0 and 2.0 mg/kg $CdCl_2$ significantly affected the body weight. Meanwhile, the highest dose group with 2.0 mg/kg $CdCl_2$ presented low fertility. Furthermore, the expression of retrotransposon mRNA was markedly increased in the higher doses group. We examined methylcytosine (mC) levels of the three active LINE-1 subfamilies TfI, A, and GfII in testis. Conclusively, Cd exposure probably undermines the male mice fertility by disrupting DNA methylation to regulate the retrotransposons. Further studies are required for identifying whether retrotransposon activation has any significant impacts on genome structure, stability, and expression in Cd-induced testicular injury, laying foundation for the treatment for male infertility.

Keywords Retrotransposons · LINE-1 · Cadmium · Testis · DNA methylation

Introduction

With the advancement of industrialization, heavy metal pollution has become a major environmental issue in modern times. Cd is a nonessential cumulative toxic metal that harms human health through contaminated foods, water, and air (Hossain et al. 2012). Once absorbed, Cd efficiently accumulates in the human body, mainly in the kidneys, where it is retained with a biological halftime around 10–30 years (Jarup and Akesson 2009). The low-level lifetime exposure to this metal may lead to damage to the kidneys, liver, skeletal system, and cardiovascular system, as well as to the deterioration of the sight and hearing (Mezynska and Brzoska 2018; Nan et al. 2020). Moreover, environmental exposure to this xenobiotic may contribute to the development of cancer of the lung, breast, prostate, pancreas, urinary bladder, and nasopharynx (Daud et al. 2009; Li et al. 2020).

Increasing research indicates that environmental exposure to Cd is related to poor semen quality and male infertility (Saygi et al. 1991). The testicles are particularly susceptible to Cd, and testicular damage can be severe even at low Cd exposure. Cd-induced reproductive toxicity involves a variety of mechanisms, including direct damage to the blood-testis barrier (Cheng and Mruk 2012; Siu et al. 2009), cytotoxicity to the Sertoli and Leydig cells (Clough et al. 1990; Lui et al. 2003; Siu et al. 2009), oxidative stress (Siu et al. 2009; Valko et al. 2005), apoptosis (Eleawa et al. 2014; Yuan et al. 2016), inflammation (Fouad et al. 2013), regulation of essential genes involved in reproductive function, and disorders of the hypothalamus-pituitary-gonadal axis (Johnson et al. 2003; Lui et al. 2003). Cd acts as a gonadal and sperm toxin when administered at a single high dose or chronic low doses. It can induce permanent and irreversible reproductive damage in animals exposed to Cd during fetal development, early life, or before puberty (Sharpe et al. 2003).

Transposable elements (TEs), as mobile repetitive sequences in genomes, comprise tandem repeats and

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interspersed repeats, which include the transposons and retrotransposons. TEs are now recognized as the driving force behind evolution and are regarded as the key regulators of gene expression.

Retrotransposons have an important role in mammalian reproduction. The activity of transposons is generally tightly controlled by epigenetic mechanisms (Waterston et al. 2002). In mice, at least three LINE-1 subfamilies (Tf, A, and Gf) retain retrotransposition activities, representing more than 9000 full-length copies in the mouse genome (Adey et al. 1991; Ostertag and Kazazian Jr. 2001). Germ cells require a stricter regulation than somatic cells for inhibiting transposon activity, and DNA methylation plays an important role in retrotransposon silencing (Ushida et al. 2012; Woodcock et al. 1997).

Few studies have investigated the relationship between Cd exposure and retrotransposon activity. An epidemiological study on 202 nonsmoking Argentine women demonstrated that DNA methylation in the peripheral blood is associated with Cd exposure in food and is related to the hypomethylation of LINE-1 (Virani et al. 2016). Kale and coworkers (Kale et al. 2005) demonstrated that environmental heavy metals trigger the retrotransposition of LINE-1. They found that Cd significantly increases the reverse transcription of LINE-1 in a dose-dependent manner.

In this study, we explored the effects of Cd exposure on the reproductive system of male mice. For the first time, we observed that the expression of specific retrotransposons increased following Cd exposure, which altered DNA methylation of LINE-1 subfamilies (TfI, A, and fII). This study reveals an essential mechanism for the pathogenesis of Cd-induced testicular injury. It provides a basis for future research for the prevention and treatment of reproductive damage in males due to Cd exposure.

Materials and methods

Animals and treatments

Eight-week-old adult male C57BL/6 J mice were raised for 1 week and then randomly divided into five groups. Each group comprises ten mice. The control group was treated with 0.9% NaCl (equivalent to receiving 0 mg/kg of body weight of $CdCl_2$), and the mice in the four treatment groups were intraperitoneally injected with $CdCl_2$ at different concentrations of 0.25, 0.5, 1.0, and 2.0 mg/kg of body weight for 35 days. $CdCl_2$ was purchased from Sigma Chemical Co. (St. Louis, USA). All the animal experiments were authorized by Institutional Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science and Technology (IACUC number: S2061).

Sampling

All mice recorded their weight daily. After 5 weeks, the mice were sacrificed by decapitation after intraperitoneal injection with pentobarbital sodium salt (Sigma), and the testes were immediately collected at − 80 °C until use. The dissected tissue of each left testis was fixed for hematoxylin–eosin staining, and the dissected tissue of each right testis was fixed for electron microscopy observations. The remaining tissues were stored at − 80 °C for biochemical analysis.

Measurement of Cd concentration in testis

Cd content in testis was detected by graphite furnace atomic absorption spectrometry (GFAAS). The testicular tissue was baked and weighed. We added nitric acid and perchloric acid to dissolve into a salt free from flowing liquid on a 280 °C hotplate for 1 h. After the samples cooled, 5 ml pure water was added to volume. The operating conditions for GFAAS require atomization at 1600 °C for Cd after a heating phase (110 °C and then 1308 °C) and pyrolysis at 500 °C for Cd. The wavelength was 228.80 nm for Cd.

Sperm quality: sperm count and motility

The sperm quality assessment was measured by the movement, shape, and vitality of the sperm. Sperm quality was studied by placing the tail of the epididymis cutting by an ophthalmic scissors in the culture medium and then maintaining at 37 °C for approximately 30 min to allow the sperm to spread freely. The sperm samples were subjected to further analyses, which included counting the motor sperm (mobile with progression and mobile without progression) and resting sperm in at least 200 sperm samples, and the results of the analyses were expressed in percentage. The total sperm count was determined by gently mixing the sperm suspension several times with a pipette and placing in a blood cell counting plate under a Olympus microscope at a final magnification of × 400.

Histological evaluation

The testicle was immediately fixed in Bouin's solution following dissection and embedded in paraffin after 48 h. The paraffin blocks were then sectioned by a Lycra paraffin slicer into 5-μm-thick sections, which were dried overnight in an incubator at 45 °C. The sections were stained with hematoxylin and eosin (HE) and finally sealed with neutral gum.

The testis tissue was fixed with glutaraldehyde and 1% osmium acid, then dehydrated with ethanol, and embedded in Epon-812. After cutting into 50–60-nm slices, slices were stained with 3% uranyl acetate-lead citrate. The ultrastructure of the testes was observed by transmission electron microscopy (TEM) (HITACHI Regulus 8100).

Assessment of testicular enzyme activity

Following dissection, the testicle was immediately frozen and stored at − 80 °C for assessing the testicular marker enzymes by molecular biology tests. The kits used for assessing the activity of lactate dehydrogenase (LDH) and alkaline phosphatase (ALP), and the levels of acid phosphatase (ACP) and γ-glutamyl transpeptidase (γ-GT), were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing).

Total RNA extraction and quantitative real-time PCR (qRT-PCR)

The total RNA was extracted from the murine testes using TRIzol Reagent (Thermo Fisher Scientific, 15596026). The RNA was reverse transcribed into cDNA, and qRT-PCR was performed using AceQ qPCR SYBR Green Master Mix (Vazyme) and a Roche Applied Science Lightcycler (Basel). Primer sequences were as follows: long interspersed element 1 type (LINE-1), F: 5'-AGTGCAGAGTTCTATCAGACCTT C-3', R: 5'-AACC TACTTGGTCAGGATGGATG-3'; short interspersed element B2 type (SINEB2), F: 5'-GAGCACCTGACTGCTCTTCC-3', R: 5'-ACACACCAGAAGAGG GCATC-3'; intracisternal A-particle element (IAP), F: 5'-CTCCATGTGCTCTGCCT TCC-3', R: 5'-CCCCGTCCCTTTTTTAGGAGA-3';and GAPDH, F: 5'-AGGTCGGTGTGA ACGGATTTG-3', R: 5'- GGGGTCGT TGATGGCAACA-3'.

LINE-1 methylation measurement

Genomic DNA was extracted using the standard phenol–chloroform methods. We retrieved consensus sequences of the active LINE-1 subfamilies in mice (Tf, A, and Gf) from Repbase. Sodium bisulfite treatments were performed using TrueMethyl Kit (Cambridge Epigenetix), according to the manufacturer's instructions. In brief, 1.5 μg DNA samples were fragmented to approximately 5 kb by sonication (Covaris). Then these DNA samples were used for bisulfite modification (bisulfite sequencing, BS). After purification using bead columns, BS samples were denatured with 50 mM NaOH at 37 °C for 30 min. Samples were incubated at 40 °C for 30 min in a thermal cycler and then centrifuged for 10 min at 14,000 G. The supernatants were removed and directly underwent sodium bisulfite treatment following the manufacturer's protocol. All reagents and columns were included in the kit (Cambridge Epigenetix). One microliter DNA sample was used for PCR amplification. The reaction mixture contained the following reagents: 1 × PCR amplification buffer (Invitrogen), 1 M betaine, 0.2 mM dNTP (TakaraBio), 3.0 mM MgCl2, 0.4 mM primers including biotin-labeled primer, 2.0 ng single-stranded DNA binding protein (Promega), and 5 U of Platinum Taq DNA polymerase (Invitrogen). PCR conditions were as follows: 3 min at 95 °C followed by 40 cycles of 10 s at 98 °C, 30 s at 58 °C, and 30 s at 72 °C for TfI and GfII and 3 min at 95 °C followed by 40 cycles of 10 s at 98 °C, 30 s at 55 °C, and 30 s at 72 °C for A. Primer sequences were as previously reported in literature (Murata et al. 2017). The PCR mixture was treated with 4 μl of Streptavidin-Sepharose beads (Amersham Biosciences) and 54 μl of × 2 binding buffer (Qiagen). Pyrosequencing was performed using a Pyromark Gold Q96 Reagents kit (Qiagen) with a PSQ 96MA instrument (Qiagen) following the manufacturer's protocols. PCR products prepared as described above were subjected to TA-cloning using TOPO TA Cloning kit (Thermo Fisher Scientific) and One Shot TOP10 Chemically Competent *E. coli* (Thermo Fisher Scientific), according to the manufacturer's instructions. After transformation, the single colonies were checked by colony PCR, and Sanger sequencing was performed by Sangon Biotech.

Statistical analyses

All the data are expressed as the mean ± standard deviation (SD). The differences among the groups were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. $P < 0.05$ was considered statistically significant, indicated by asterisks.

Results

General growth and toxicity profile following Cd exposure in male mice

The general growth of the mice in the control group was well. They had a well-proportioned build, soft silky fur, and healthy teeth. However, the growth trend of the mice that were exposed to Cd was slow, and the mice that received the maximum dose of Cd (2.0 mg/kg of body weight) had rough yellow coats, and their body weights were significantly lower than those of the mice in the control group (Fig. 1a). The groups did not exhibit any significant difference with respect to food and water intake, and there were no obvious symptoms of Cd poisoning in any of the groups.

Cd exposure led to the accumulation of Cd in the testis. As depicted in Fig. 1b, when mice are exposed to lower cadmium concentrations, the body has a specific metabolic capacity. After being treated with Cd for 5 weeks, the Cd concentration in the testis in the three groups that received CdCl2 at doses of 0.5, 1.0, and 2.0 mg/kg of body weight were increased significantly compared with the control group.

Effects of Cd exposure on the testicular tissues of male mice

Cd decreased the activity of the germ cell marker enzymes LDH and ALP and the Sertoli cell marker enzymes ACP

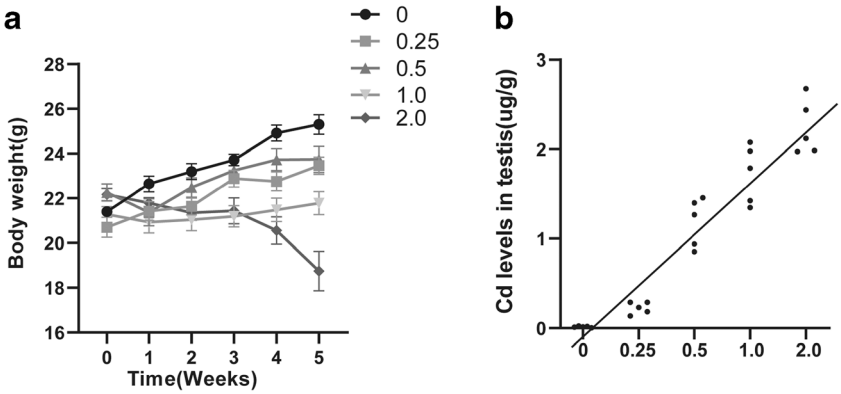

Fig. 1 General growth and cadmium concentration in testis following Cd exposure in male mice. **a** Changes in the body weights of male mice treated with different concentrations of $CdCl_2$ for 5 weeks. **b** Cd concentration in testis of all Cd-treated male mice increased significantly compared with the control group. All the data are represented as the mean ± SD ($n = 5$). Compared with the control $^*P < 0.05$

and γ-GT in a time-dose-dependent manner. The level of LDH in the group that received 2.0 mg/kg of $CdCl_2$ was significantly decreased ($P < 0.05$) than that of the other groups (Fig. 2a). The activity ACP in larger doses group with $CdCl_2$ at 1.0 mg/kg and 2.0 mg/kg was significantly altered ($P < 0.05$) in comparison to that of the control group (Fig. 2b). The levels of ACP and γ-GT were decreased in the three groups with 0.5, 1.0, and 2.0 mg/kg $CdCl_2$ (Fig. 2c, d). The activities of both ACP and γ-GT were found to be more sensitive to Cd exposure than the control group.

The testicular morphology of each group was evaluated by HE staining following paraffin embedding. The testicular tissue sections of the control group exhibited normal histology (Fig. 3a). The seminiferous tubules were generally seen as multiple layers of cells in the seminiferous epithelium. Each seminiferous tubule contained germ cells at different stages of development. There were pathological changes in the seminiferous tubules in the Cd-treated groups. High doses of $CdCl_2$ decreased the number of cell layers in the seminiferous tubules, disrupted the arrangement of the germ cells, and presented noticeable vacuoles in the cytoplasm of the Sertoli cells and the gap between the cells in comparison to that of the control group (Fig. 3a). Transmission electron microscopy was performed to examine the effects of Cd exposure in murine testes. The ultrastructure of the seminiferous tubule demonstrates obvious apoptosis and necrosis of many germ cells

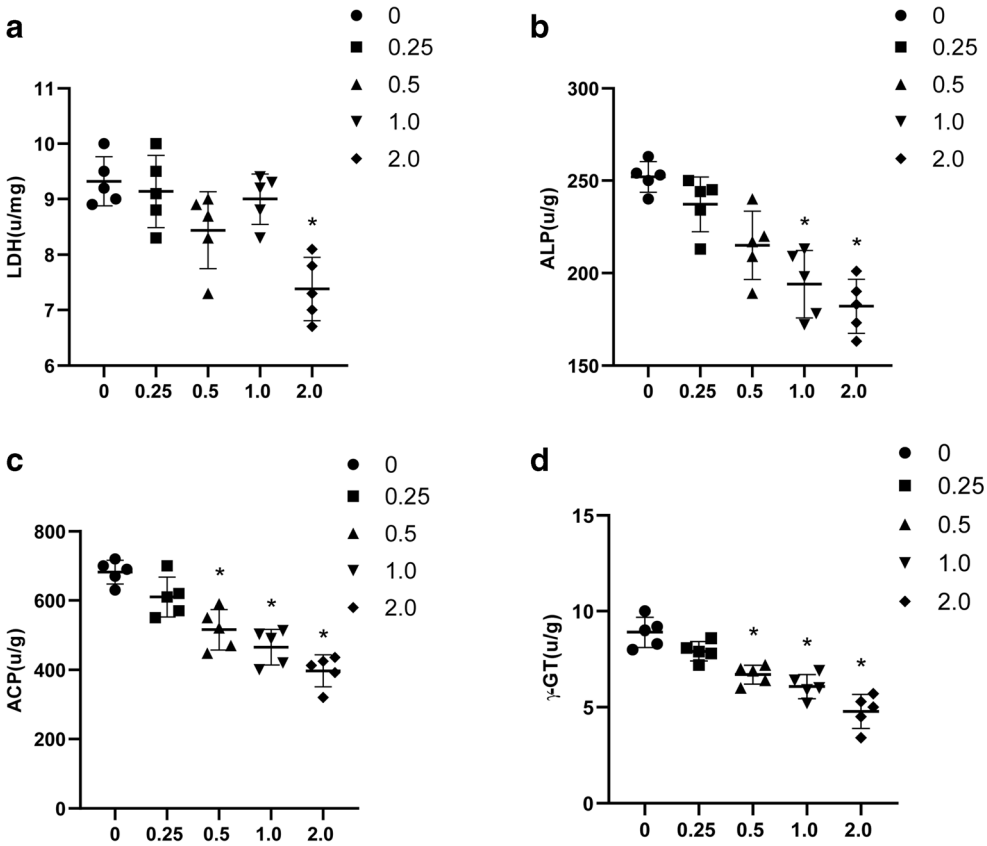

Fig. 2 Changes in the activity of testicular enzymes following Cd exposure in male mice. **a** LDH and **b** ALP are markers of germ cells; **c** ACP and **d** γ-GT are markers of Sertoli cells. All the data are represented as the mean ± SD ($n = 5$). Compared with the control $^*P < 0.05$

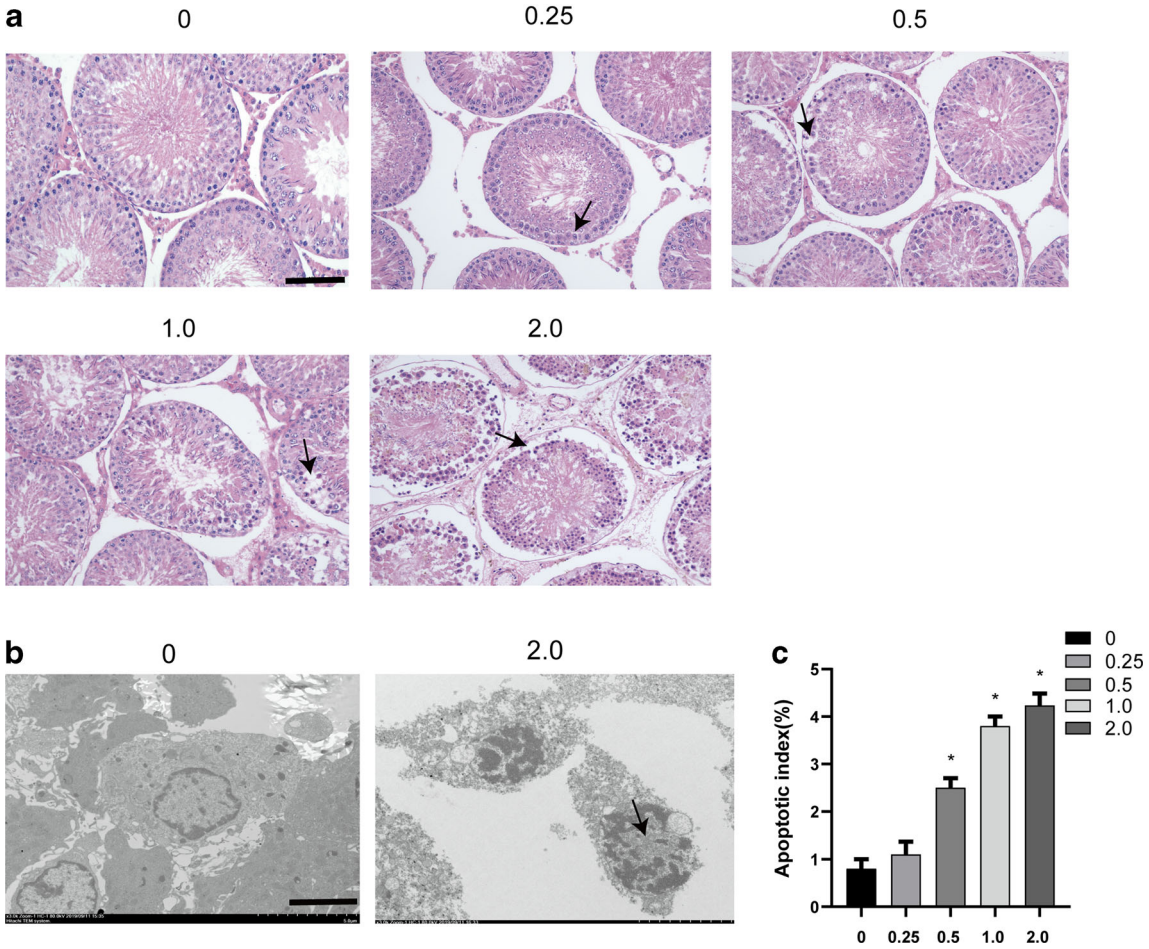

Fig. 3 Effects of Cd on testicular histology in male mice treated with Cd for 5 weeks. **a** There were 5 groups that received $CdCl_2$ at doses of 0, 0.25, 0.5, 1.0, and 2.0 mg/kg of body weight. The arrows indicate the abnormal spermatogenic tubules in the Cd-treated group. The scale bar represents 50 μm. **b** Effect of 2.0 mg/kg $CdCl_2$ exposure on the ultrastructure of testis was detected by transmission electron microscopy. Arrows indicate apoptotic cells in the testis. The scale bar represents 500 nm. **c** Apoptotic index. All data are expressed as the mean ± SD ($n = 6$). Compared with the control $^*P < 0.05$

in the highest dose group (Fig. 3b). We counted the number of necrotic/apoptotic germ cells in each group and calculated apoptotic index. The number of necrotic/apoptotic germ cells was significantly increased in the three groups with $CdCl_2$ at doses of 0.5, 1.0, and 2.0 mg/kg.

Effects of Cd exposure on sperm quality

The sperm count and sperm motility in the Cd-treated mice are depicted in Fig.4. The sperm motility in the groups exposed to Cd decreased significantly after 5 weeks of Cd exposure compared to that of the control group. The sperm counts in the groups that received 1.0 and 2.0 mg/kg $CdCl_2$ were significantly reduced by approximately 30% in comparison to that of the control group, and the number of abnormal sperms increased in both the Cd-treated groups (Fig. 4a). Also, Cd reduced sperm motility in a dose-dependent manner. (Fig. 4b).

Cd exposure affected retrotransposon expression in the testes

Exposure to environmental toxins may affect the interactions of retrotransposons with the genome, increasing the effect of transposable elements. The expression of the retrotransposons LINE-1, SINE B2, and IAP in the testes was examined in this study. The results demonstrated that the expression of the retrotransposons, LINE-1, and SINE B2 was significantly up-regulated in the groups with 0.5 mg/kg or higher $CdCl_2$ (Fig. 5a, b). However, there was no significant change in the mRNA levels of IAP in each of the groups (Fig. 5c).

Cd exposure affected global DNA methylation in male mice

At least three LINE-1 subfamilies (Tf, A, and Gf) retain retrotransposition activities in mice, representing more

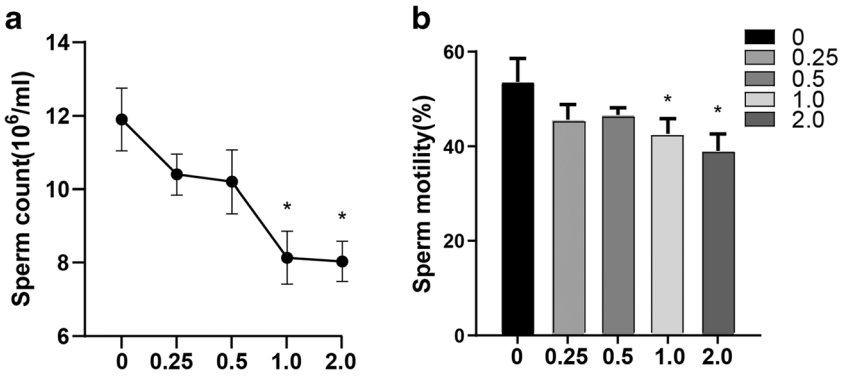

Fig. 4 Effects of Cd exposure on the sperm quality of male mice. **a** Sperm counts of the male mice exposed to Cd for 5 weeks. **b** Sperm motility. All the data are represented as the mean ± SD ($n = 5$). Compared with the control $^{*}P < 0.05$, $^{**}P < 0.01$

than 9000 full-length copies in the mouse genome. Consensus sequences of active LINE-1 subfamilies in mice (Tf, A, and Gf) were retrieved from Repbase. Pyrosequencing assay for TfI, A, and GfII was characterized in a previous study (Murata et al. 2017). We performed pyrosequencing-based DNA methylation assays of the 5'-UTR in the active LINE-1 subfamilies TfI, A, and GfII, and we evaluated methylation levels of the testis (Fig. 6). Interestingly, we found that methylation levels in several subfamilies of line decreased with increasing exposure to Cd in the testis. We examined two CpG sites on LINE-1 subfamilies TfI, with decreased methylation at site 1 and decreased methylation at site 2 only in the highest dose group. The methylation level of LINE-1 subfamilies A decreased and showed a statistical difference in the group with higher Cd treatment dose. For LINE-1 subfamilies GfII, we examined three sites, there was a significant decrease in methylation level at site 3, but no significant difference was observed at other sites. Results suggested that the exposure of male mice to Cd can reduce the DNA methylation levels of LINE-1 in the testes, which can also be responsible for the increase in the transcription levels of retrotransposons.

Discussion

We established a chronic animal model to mimic environmental Cd exposure on human and reported that hypomethylation of LINE-1 retrotransposons was associated with Cd-induced testicular injury. The choice of Cd exposure dose is determined on the basis of preliminary exploration experiments in the laboratory and with reference to relevant literature (Du et al. 2015; Oliveira et al. 2012). Our study revealed Cd exposure for 5 weeks resulted in the continuous accumulation of Cd in the testes, which affected the body weight and decreased the activity of testicular marker enzymes. Then, the morphology of testicular tissue was damaged, resulting in a significant decrease in sperm count and motility. Testicular marker enzymes include LDH, ALP, ACP, and γ-GT. LDH is a germ cell-specific enzyme that plays a vital role in meiotic energy metabolism (Zhong and Kleene 1999). ALP is associated with the division of spermatogenic cells in murine testes. ACP mainly exists in the cytoplasm of SCs and functions on the degeneration of seminiferous epithelium (Yang et al. 2010). γ-GT plays a role in SCs and promotes sperm maturation (Thompson et al. 2015). In this study, Cd abated LDH, ALP, ACP, and γ-GT, manifesting that Cd damaged testicular cells, including germ cells and SCs. The trend of testicular

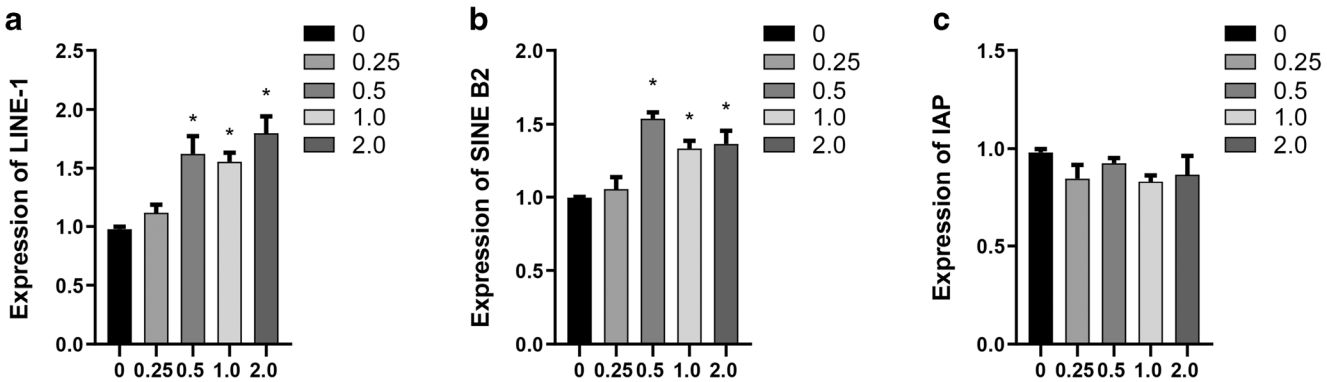

Fig. 5 Cd exposure affected the expression of retrotransposons in the testes. QRT-PCR analysis of LINE-1 (**a**), SINE B2 (**b**), and IAP (**c**) in male mice treated with Cd for 5 weeks. All the data are represented as the mean ± SD ($n = 5$). Compared with the control $^{*}P < 0.05$

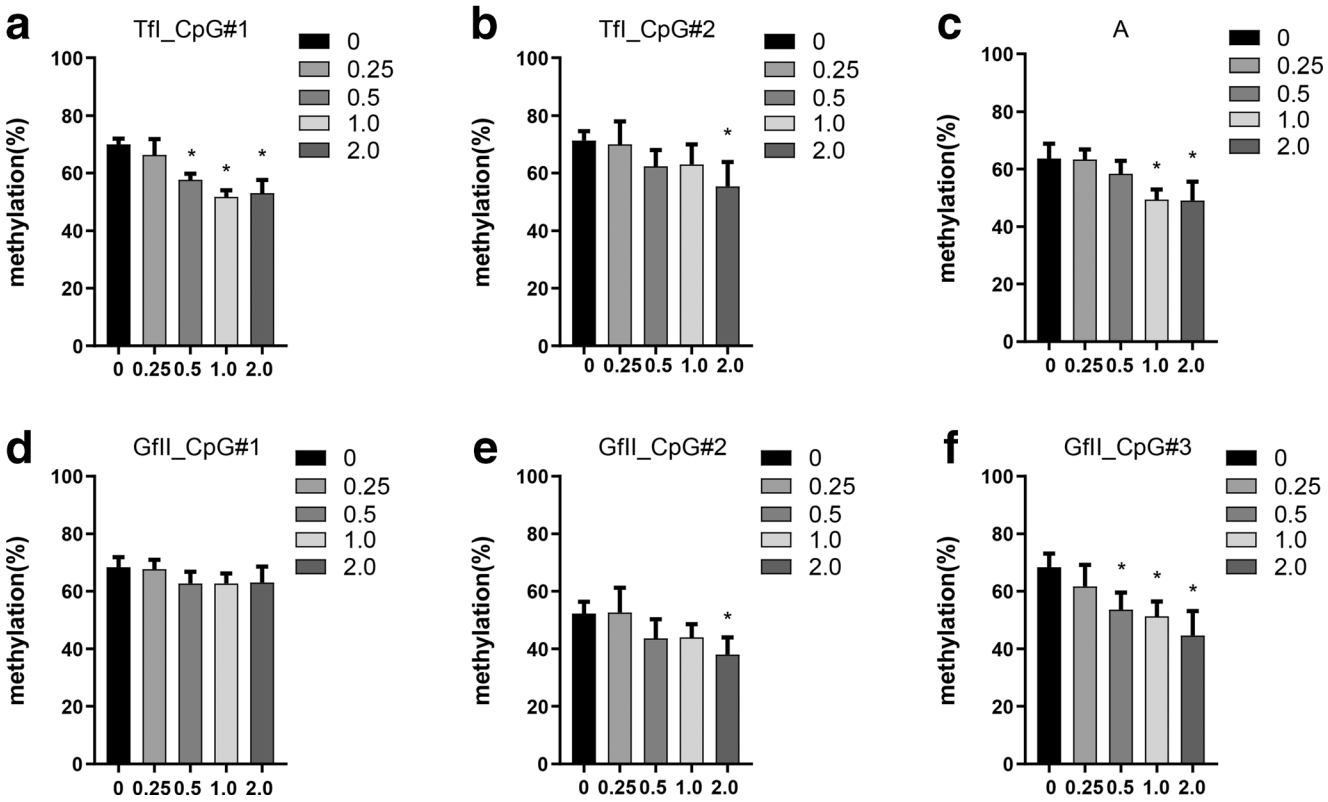

Fig. 6 Cd exposure affected DNA methylation levels of the active LINE-1 subfamilies in testis. For TfI, two CpG sites located within the nonmonomer region; for A, one CpG site located within the nonmonomer region; and for GfII0, three CpG sites (two located within the truncated monomer, and one within the nonmonomer region) were analyzed. Compared with the control $*P < 0.05$

histomorphological changes was noticeable. With the increase of Cd exposure, the damage degree of the spermatogenic epithelium in the testicular convoluted fine duct showed a dose-effective-increasing trend. The apoptotic index also increased significantly.

Then, we examined the expression of some retrotransposons and found that LINE-1 and SINE B2 mRNAs were markedly increased in the groups exposed to CdCl2 at doses of 0.5, 1.0, and 2.0 mg/kg of body weight. Alterations in DNA methylation may play a potentially important role. Performing pyrosequencing-based DNA methylation assays of LINE-1 subfamilies TfI, A, and GfII in testis, we found that the methylation of CpG sites in several LINE-1 subfamilies was decreased, which may be responsible for the increase in LINE-1 expression levels.

The CpG sites of the LINE-1 promoter are usually highly methylated, and the genome-wide methylation of these sites plays a critical role in cell proliferation and differentiation and tumorigenesis (Cho et al. 2010; Choi et al. 2009). The modes of regulation and biological functions of retrotransposons in germline cells are different from those of other genes in the genome (Goodier 2016; Kelleher 2017). Alterations in DNA methylation induces modifications in retrotransposons that affect the organism itself, and these alterations can also be transmitted to the offspring through the germline cells (Dolinoy et al. 2007; Ho et al. 2006).

Numerous studies have increasingly demonstrated that Cd exposure alters the epigenetic marks, which may explain the possible relation between the heritable changes in gene expression and disease susceptibility (Demanelis et al. 2017; Gadhia et al. 2015). However, few studies have investigated the effects of Cd exposure on retrotransposon expression by epigenetic studies (DNA methylation). Our study first reported the changes in the expression of retrotransposons following testicular Cd exposure.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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