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ORIGINAL ARTICLE ***Reproductive biology***

Transgenerational impaired male fertility with an *Igf2* epigenetic defect in the rat are induced by the endocrine disruptor *p,p′*-DDE

Yang Song*, Nanxiang Wu*, Simeng Wang, Ming Gao, Peng Song, Jianlin Lou, Yufeng Tan, and Kecheng Liu

Hygiene Institute in Zhejiang Academy of Medical Sciences, 182 Tianmushan Road, Hangzhou 310013, China

*Correspondence address. Tel: +86-57188215594; Fax: +86-57188085911; E-mail: sygp_0@163.com (Y.S.); wunanxiang@hotmail.com (N.W.)

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STUDY QUESTION: What are the epigenetic mechanisms underlying the transgenerational effect of *p,p′*-DDE on male fertility?

SUMMARY ANSWER: Impaired male fertility with an *Igf2* epigenetic defect is transgenerationally inherited upon exposure of *p,p′*-DDE.

WHAT IS KNOWN ALREADY: *p,p′*-Dichlorodiphenoxydichloroethylene (*p,p′*-DDE) is one of the primary metabolite products of the ancestral organochlorine pesticide dichlorodiphenoxytrichloroethane. As it is a known anti-androgen endocrine disruptor, it could cause harmful effects on the male reproductive system.

STUDY DESIGN, SIZE, DURATION: Pregnant rats (F0) were administered with *p,p′*-DDE or corn oil at the critical time of testis development, i.e. from gestation days 8 to 15. Male and female rats of the F1 generation were mated with each other to produce F2 progeny. To reveal whether the transgenerational phenotype is produced by the maternal or paternal line, F3 progeny were generated by intercrossing control (C) and treated (DDE) males and females of the F2 generation according to the following groups: (i) C♂-C♀, (ii) DDE♂-DDE♀, (iii) DDE♂-C♀ and (iv) C♂-DDE♀.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Mature sperm and testes were collected from male offspring of the F1–F3 generations for the examination of male fertility parameters, i.e. sperm count and motility, testis histology and apoptosis. Expression of the imprinted genes, *H19* and *Igf2*, was detected by real-time PCR. *Igf2* DMR2 methylation was analyzed by bisulfite genomic sequencing.

MAIN RESULTS AND THE ROLE OF CHANCE: Upon exposure of *p,p′*-DDE, the male F1 generation showed impaired male fertility and altered imprinted gene expression caused by *Igf2* DMR2 hypomethylation. These defects were transferred to the F3 generation through the male germline.

LIMITATIONS, REASONS FOR CAUTION: This study has examined the effect of *p,p′*-DDE only on the sperm number and motility and the possible mechanism of *Igf2* DMR2 methylation *in vivo* and thus has some limitations. Further investigation is necessary to focus on the epigenetic effects of *p,p′*-DDE at the genome level and to include a more detailed semen quality analysis including sperm morphology assessment.

WIDER IMPLICATIONS OF THE FINDINGS: Impaired male fertility with epigenetic alterations is transgenerationally inherited after environmental exposure of *p,p′*-DDE, posing significant implications in the etiology of male infertility.

STUDY FUNDING/COMPETING INTEREST(S): The present research was supported by National Natural Science Fund for Young Scholar (81102161), the Natural Science Fund of Zhejiang Province (LY14H260004) and funding from the Health Department of Zhejiang Province (201475777). No competing interests are declared.

Key words: *p,p′*-DDE / male fertility / transgeneration / *Igf2* / methylation

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Introduction

Since the 1990s, various studies (Carlsen *et al.*, 1992; Swan *et al.*, 2000; Rolland *et al.*, 2013) have reported that young males in developed or industrialized countries have decreased sperm concentrations, accounting for the fertility decline currently observed (Jensen *et al.*, 2008; Joensen *et al.*, 2009). Although the factors causing these deviations are under debate, data from toxicology and epidemiology studies have suggested that endocrine disruptor chemicals (EDCs) can have serious effects on male gonadal function (Roeleveld and Bretveld, 2008; Safe, 2013).

p,p′-dichlorodiphenoxydichloroethylene (*p,p*′-DDE) is one of the primary metabolite products of the ancestral organochlorine pesticide, dichlorodiphenoxytrichloroethane (DDT), and has the strongest persistence in the environment. It is also a representative EDC commonly found in human tissues (Walter and Aimin, 2005). Although environmental levels of *p,p*′-DDE have been diminishing over recent decades, an elevated burden may still be accumulated in the general population through diet or other pathways. Furthermore, it is important to note that *p,p*′-DDE may be transferred through cord blood, the placenta or breast milk to the offspring (Dewan *et al.*, 2013; Porpora *et al.*, 2013), inducing male reproductive defects such as gonad abnormalities (Kelce *et al.*, 1995) and reduced sperm number (Loeffler and Peterson, 1999).

Epigenetic transgenerational inheritance involves epigenetic defects being transmitted to subsequent generations through the germline (sperm or oocyte) without direct hazardous exposures (Jirtle and Skinner, 2007, 2011). Growing numbers of studies have reported that EDCs may induce epigenetic effects on the germline, which could possibly be the reason for the transgenerational impairment of male fertility. DDT may cause transgenerational effects on the testis due to epimutations of differentially methylated regions (DMRs) in the sperm (Skinner *et al.*, 2013a,b). Vinclozolin may impair male fertility transgenerationally by defective DNA methylation in the male germline (Anway *et al.*, 2005), and male fertility has been observed to be decreased in subsequent generations after a transient exposure to BaP (Mohamedel *et al.*, 2010). These studies indicate that epigenetic modifications induced by environmental factors may play an important role in impaired fertility.

Genomic imprinting is a specific epigenetic phenomenon which regulates genes expression based on whether the allele is of paternal or maternal origin. It is controlled by methylation of specific sites called DMRs (Li *et al.*, 1993; Bartolomei and Tilghman, 1997; Reik and Murrell, 2000), which is established in the germline and maintained in the somatic cells. During fetal development, imprinting reprogramming (erasure and re-establishment) occurs in the germ cells which will give rise to the F2 generation (Davis *et al.*, 2000), which means there are possible effects on the later development of human disease (Tremblay and Hamet, 2008). Furthermore, if these changes somehow successfully escape reprogramming in the next generation, minimal imprinting defects would be transgenerationally transmitted, causing serious phenotypes due to the changes being preserved (Stouder and Paoloni-Giacobino, 2010). The paternally transcribed *Igf2* gene and the maternally transcribed *H19* gene are two critical imprinted genes with possible effects on spermatogenesis. Recent studies have shown hypomethylation of the *Igf2–H19* imprinting control regions (ICR) in spermatozoa of men with oligozoospermia (Marques *et al.*, 2008; Poplinski *et al.*, 2010). Whether this imprinting defect could be involved in the transgenerational impairment of male fertility caused by *p,p*′-DDE is not clear.

In this study, we hypothesized that a transient exposure to the endocrine disruptor *p,p*′-DDE could impair male fertility by altering imprinting. To reveal the possible mechanism underlying the transgenerational phenotype, we quantified the expression of the specific imprinted genes *Igf2* and *H19* and the percentage of methylation at the *Igf2* DMR in the sperm of three successive generations.

Materials and Methods

Chemicals

p,p′-DDE, bovine serum albumin (BSA) and human tube fluid medium were purchased from Sigma Chemical Co. (St Louis, USA). The Tunel apoptosis detection kit, hotStar Taq polymerase, SYBR Green PCR Master Mix and pMD19-T-simple cloning vector were purchased from TAKARA Biotechnology (Dalian) Co. (Dalian, Ltd., China). The ELISA kit was purchased from R&D Co. (Abingdon, Oxon, UK). RNeasy was purchased from Qiagen Co. (Valencia, CA). The DNA extraction kit was purchased from Beijing CoWin Bioscience Co. (Beijing, China). The EZ DNA Methylation-Gold Kit™ was bought from Zymo research biotechnology company (USA). The Methyl Flash™ Global DNA Methylation Quantification Kit was purchased from Epigentek Co. (Brooklyn, NY , USA). All reagents for the reverse transcription were purchased from Promega Co. (Madison, WI, USA).

Animals and treatments

Timed-pregnant Sprague–Dawley rats were obtained from the experimental animal center in Zhejiang Province. All animals were housed in a room in a controlled temperature (22–25°C) and light cycle of 12 h and provided freely with sterile water and AIN-93G rodent ration. After cohabiting with male rats overnight, female rats were determined to be pregnant by observation of a copulation plug (gestation day (GD) 0). From GD 8 to GD15, the pregnant rats were administrated by gavage with 100 mg/kg of body weight of *p,p*′-DDE or corn oil as the vehicle control. At spontaneous parturition, we reduced the litter size of the F1 generation to 10 to assure uniformity. The F2 generation was generated by mating male and female rats of the F1 generation The F3 generation was obtained by intercrossing the control and treated male and females of the F2 generation according to the following groups: (i) C♂-C♀, (ii) DDE♂-DDE♀, (iii) DDE♂-C♀ and (iv) C♂-DDE♀. No inbreeding or sibling crosses were generated. All procedures were performed according to Zhejiang Academy of Medical Sciences Animal Care and Use Committee.

The numbers of animals in the experiments (i.e. *n* values) were as follows: F1 control 20, F1 treated 20; F2 control 20, F2 treated 22; F3 control 20, F3 DDE♂-DDE♀ 13, F3 DDE♂-C♀ 20 and F3 C♂-DDE♀ 20. All adult males were injected with 1.2 ml of 10% chloral hydrate in the left lower quadrant of the abdominal cavity and decapitated at post-natal day 120.

Sperm number and motility analysis and mobile sperm collection

Mature sperm were collected according to Tayama *et al.* (2006). The rat vas deferens and epididymis were separated, trimmed free of fat, placed into a Petri dish and scored with a razor blade in human tube fluid medium containing 0.1% BSA at 37°C for 30 min to allow sperm to diffuse into the medium. The medium was then pipetted into a pre-warmed (37°C) microcell slide chamber placed on a warm stage attached to a microscope. Then sperm number and motility was analyzed with the computer-aided sperm analysis (CASA, Hamilton Thorne, Danvers, MA, USA) according to Mortimer *et al.* (1998). The motile percentage was recorded as well as the average path velocity (VAP), linearity % (LIN%), straight-line velocity (VSL), curvilinear velocity (VCL) and amplitude of lateral head displacement (ALH).

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The swim-up technique was used to collect the motile sperm. The supernatant containing the motile sperm fraction was collected after sedimentation of the sperm suspension at 37°C for 30 min. After repeating this three times, the sperm were centrifuged at 2800*g* for 3 min and collected.

Histology

Following a weight determination, testes were submerged in modified Davidson's fluid according to Latendresse *et al.* (2002), paraffin-embedded, sectioned and stained with H&E (hematoxylin and eosin) for morphological analysis.

Spermatogenic cell apoptosis detection with Tunel

To detect the apoptosis of spermatogenic cells at the single cell level, the *in situ* cell death detection kit was used according to the manual. After being deparaffinized with xylene and rehydrated, the testicular sections were treated with 20 μg/ml proteinase K. The Tunel reaction mixture containing enzyme TdT and digoxygenin-labeled dUTP was added to the 3′-hydroxyl ends of DNA. After the enzyme and coloration reaction, brownish staining apoptotic cells were counted at 200× magnification in each testis section. The apoptotic index (AI) was calculated as the number of apoptotic cells number divided by the total cell number per section. Furthermore, the apoptotic germ cells at various stages were analyzed according to Hess and Renato (2008). A total of three rats per group were analyzed.

Serum testosterone measurement

Whole blood from abdominal aorta was prepared from male adults, allowed to coagulate and then centrifuged at 4°C for 30 min. Serum testosterone was assayed in duplicate by ELISA kits according to the manual. All serum samples

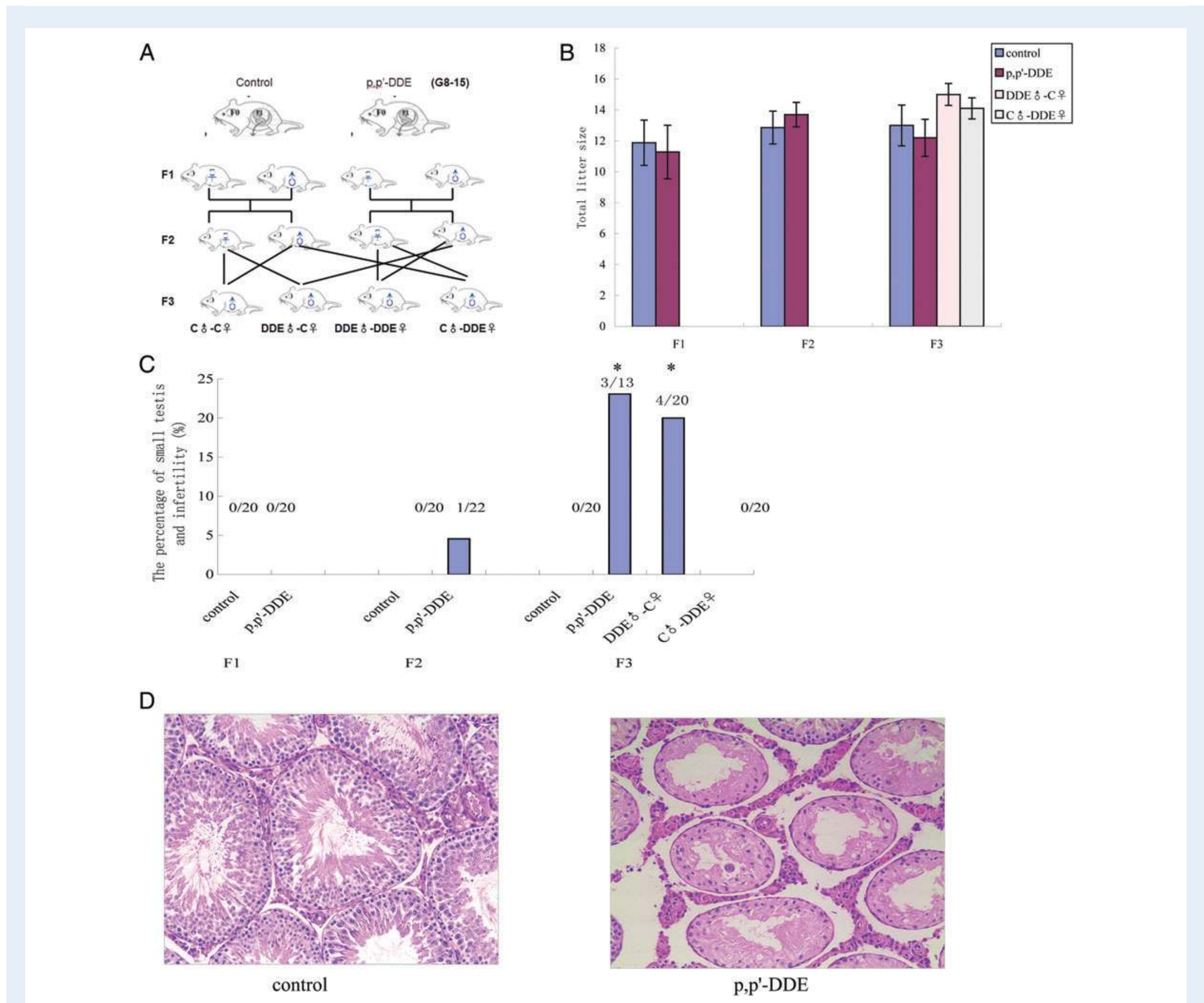

Figure 1 Transgenerational phenotype after a transient *p,p*′-DDE treatment. (**A**) Transgenerational experimental design of the animal model after a transient exposure to *p,p*′-DDE. (**B**) The litter size of fertile males after a transient exposure to *p,p*′-DDE ($n = 6$–8 litters/group). (**C**) The percentage of small testis and rate of infertility in the F1–F3 generations of male offspring after a transient exposure to *p,p*′-DDE. (**D**) Testis histology in control and infertile male rats of the F3 generation. Results are expressed as means ± SE. *$P < 0.05$. Significance was determined by Student's *t*-test in F1 and F2 offspring and ANOVA in F3 offspring.

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were added in the well pre-coated with enzyme-linked testosterone antibody. After the coloration reaction, the product was quantified by a microplate reader.

Quantitative real-time PCR analysis of Igf2 and H19

After being extracted from sperm according to Chomczynski and Sacchi (1987), RNA was reverse transcripted with Oligo (dT) primers and an RT enzyme mix. The cDNA product was diluted four times and reacted with SYBR® Premix Ex Taq™ and separate primers as follows:
Igf2 forward primer: 5′-TAACACCGGCTAGAGCCATCAAC-3′
Igf2 reverse primer: 5′-ACTCAGGCCAAGCGATAGAGACA-3′
H19 forward primer: 5′-GCATTCTAGGCTGGGGTCAA-3′
H19 reverse primer: 5′-TGCCCTTCTTTCCCGCTCT-3′
β-actin forward primer: 5′-GGAGATTACTGCCCTGGCTCCTA-3′
β-actin reverse primer: 5′-GACTCATCGTACTCCTGCTTGCTG-3′.
PCR was performed with 7.2 μl RNase-free water, 0.3 μl 10 μM forward and reverse primers and 10 μl SYBR Green PCR Mix. As soon as the reaction was complete, an additional cycle was in progress to obtain the dissociation curves to test the specificity of the PCR products. The expression of imprinted gene was relatively quantified with $2^{-\Delta\Delta Ct}$ according to the manual.

Methylation status of Igf2 DMR2 in sperm with bisulfite genomic sequencing

Methylation of *Igf2* DMR2 was evaluated by bisulfite sequencing PCR. Briefly, sperm DNA was extracted with a DNA extraction kit according to Guerrero-Bosagna *et al* (2010) and modified with the EZ DNA Methylation-Gold Kit™ to deaminate cytosine to uracil. The modified DNA was PCR amplified with HotStar Taq polymerase. The forward and reverse primers were GAAATTTGTTGATTAGTATTTTTTTT and TACCACATAATTTAATTCACTAATAATTAC. Thermal cycling conditions were 95°C for 10 min, 40 cycles of 95°C/30 s, 58°C/30 s and 72°C/50 s, 5 min at 72°C. The PCR product was gel purified and cloned into the pMD19-T-simple cloning vector. Ten clones from each sample were sequenced on the ABI Prism DNA sequence version 3.7 and sequenced data were analyzed by BiQ analyzer.

Global methylation status in sperm

The global methylation status in sperm DNA was analyzed with Methyl Flash™ Global DNA Methylation Quantification Kit as the manufacturer. Different concentrations of methylated DNA controls and 100 ng samples of sperm DNA were added in duplicates. The absorbance was read on a microplate reader at 450 nm after the addition of the primary antibody (1 mg/ml), 5-methy-cytosine (5-^{m}C) and secondary antibody (400 mg/ml). The optical density values were plotted against the amount of methylated DNA in control standards, the slope of the plot was determined and then the amount of methylated DNA in samples was calculated using the formula:

$$5\text{–}^{m}\mathrm{C}\,(ng) = \frac{Sample\ OD - blank\ OD}{\mathrm{Slope} \times 2}$$

$$5\text{–}^{m}C\% = 5\text{–}^{m}\mathrm{C}\,(\mathrm{ng}) \times \frac{100\%}{100}$$

Statistical analysis

Data were reported as mean and standard error. Differences were determined by independent samples *t*-test or Pearson's χ^2 test or ANOVA with SPSS (Version 12.0, USA).

Table I. Transgenerational decreased sperm number and motility after p,p'-DDE treatment.

	n	Total (million/ml)	Motile (%)	VAP	VSL	VCL	ALH	LIN%
F1								
Control	8	70.63 ± 4.17	76.75 ± 2.68	216.40 ± 13.19	158.71 ± 11.27	368.19 ± 13.92	14.05 ± 0.52	44.50 ± 1.05
DDE	8	50.00 ± 4.62*	62.42 ± 3.32*	153.27 ± 12.88*	101.52 ± 5.82*	297.86 ± 28.16*	11.44 ± 0.84*	38.57 ± 1.64*
F2								
Control	7	82.86 ± 14.59	82.14 ± 2.43	248.21 ± 12.49	162.07 ± 6.52	519.23 ± 35.40	17.51 ± 0.98	32.14 ± 1.53
DDE	8	43.75 ± 8.85#	60.88 ± 8.85#	179.23 ± 26.44#	116.80 ± 17.36#	389.41 ± 58.46	14.14 ± 2.13	28.38 ± 4.18
F3								
C♂-C♀	9	68.89 ± 13.79	68.56 ± 3.56	203.57 ± 11.93	135.40 ± 6.89	411.17 ± 21.04	16.54 ± 0.88	35.44 ± 0.41
C♂-DDE♀	11	55.50 ± 13.32	67.20 ± 4.68	178.73 ± 21.97	119.28 ± 15.47	358.34 ± 44.14	14.98 ± 1.79	32.40 ± 4.08
DDE♂-C♀	5	28.00 ± 11.90‡	34.6 ± 14.13‡	149.90 ± 40.78‡	98.46 ± 26.51‡	301.9 ± 80.77‡	14.16 ± 3.71	27.80 ± 7.02
DDE♂-DDE♀	6	21.66 ± 10.14‡	29.33 ± 13.35‡	95.93 ± 43.13‡	63.37 ± 29.18‡	152.53 ± 78.28‡	10.07 ± 5.08	15.83 ± 7.38‡

Data are means ± SE.
*$P < 0.05$ versus F1 control, #$P < 0.05$ versus F2 control, ‡$P < 0.05$ versus C♂-C♀.
Significance was determined by Student's *t*-test in F1 offspring, F2 offspring and ANOVA in F3 offspring. ALH, amplitude of lateral head displacement; LIN%, linearity %; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity.

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Results

Decreased sperm number and motility were transmitted transgenerationally via the male germline after a transient exposure to *p,p′*-DDE

We established a transgenerational animal model after a transient exposure to *p,p′*-DDE during pregnancy. To reveal whether the male or female germline produced the transgenerational phenotype, a treated F2 generation was generated by inbreeding males and females of the treated F1 generation. The F3 generation was obtained by inter-crossing the control and treated males and females of the F2 generation (Fig. 1A).

Table 1 shows that sperm number and motility declined for three successive generations after a transient treatment with *p,p′*-DDE during the critical period of gonad development. As for the motility parameters, VAP and VSL decreased. The outcross experiment revealed that sperm number and motility decreased in DDE♂-C♀ and DDE♂ mice but there was no effect in C♂-DDE♀ mice, indicating that the male germline carried the transgenerational decrease in sperm quality.

To determine whether the decreased sperm number and quality affected male fertility, the litter size was analyzed in all tested generations. No difference in litter size could be observed in all treated males that were fertile (Fig. 1B). However, we found a link between male infertility and small testis size (Fig. 1C). Reductions in testes size occurred in 1 out of 22 treated F2 generation animals, and in 3 and 4 out of 13 and 20 exposed F3 generation of the DDE♂-C♀ and DDE♂-DDE♀ groups. The pathological section of a representative small testis is shown in Fig. 1D, indicating the abnormal seminiferous tubule morphology without elongated spermatids.

Apoptosis of spermatogenic cells was transgenerationally induced through the male germline after a transient exposure to *p,p′*-DDE

Spermatogenic cell apoptosis was analyzed in testis cross sections from each generation. Figure 2A shows the brownish labelings of apoptotic cells in the treated F1 generation, compared with such cells in the control. The majority of apoptotic testicular cells were primary spermatocytes along the periphery of the seminiferous tubules, although some were spermagonia. No apoptosis could be observed in Sertoli and interstitial cells.

Figure 2B demonstrates that spermatogenic cell apoptosis increases more than 2-fold than in the treated F1 and F2 generations compared with the controls. As for the F3 offspring, considerable apoptosis happened in the testes of DDE♂-C♀ and DDE♂-DDE♀ mice, no apoptotic effect in the testes of C♂-DDE♀ mice.

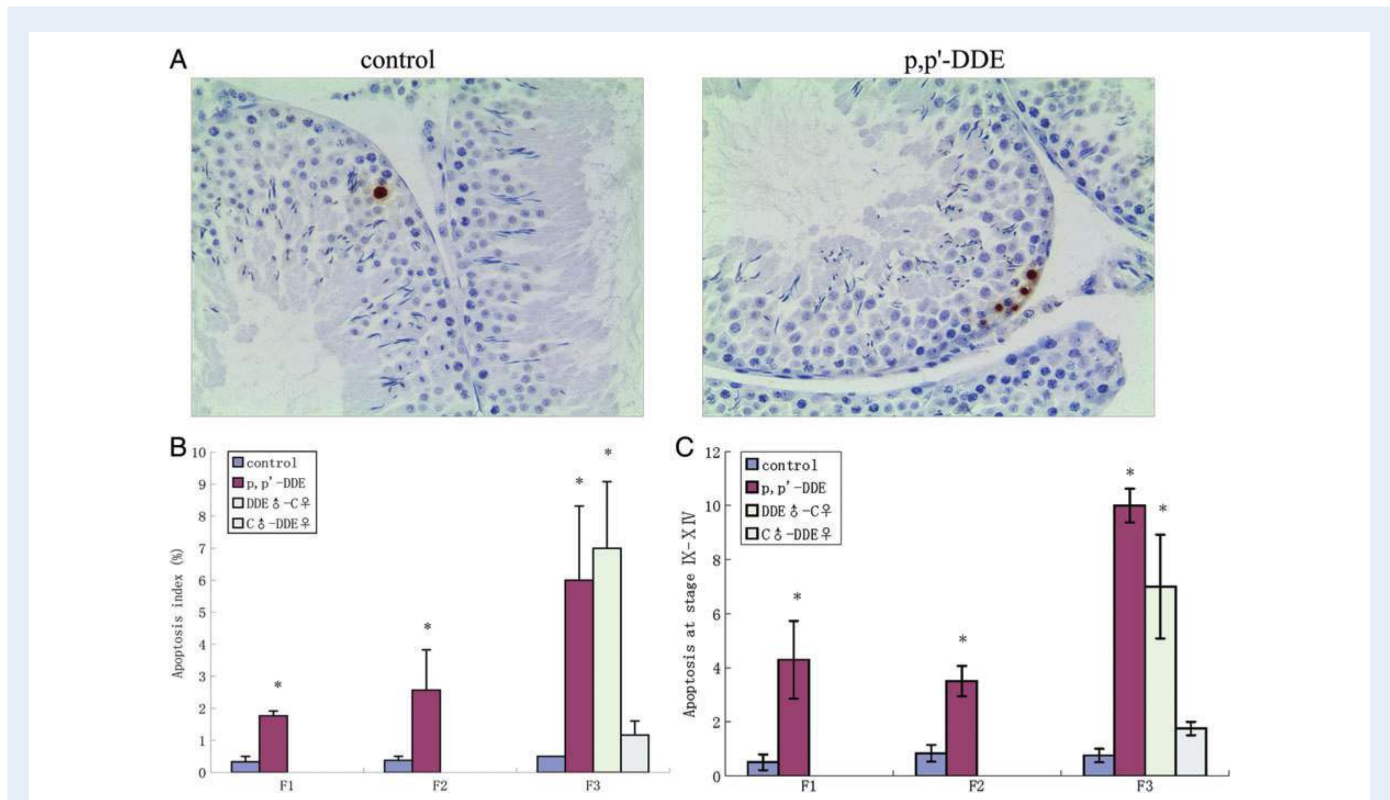

Figure 2 Transgenerational apoptosis induced after a transient *p,p′*-DDE treatment. (**A**) Testicular apoptosis in F1 generation after *p,p′*-DDE treatment. More apoptotic cells with brownish staining were located along the basement of seminiferous tubules after treatment with *p,p′*-DDE compared with few apoptotic cells in control. Representative illustrations were shown at 200× magnification. (**B**) The testicular apoptosis index in the F1–F3 generation male offspring ($n = 3$ rats/group). (**C**) Apoptotic germ cell number at stages IX–XIV in F1–F3 generation male offspring ($n = 3$ rats/group). *$P < 0.05$. Significance was determined by Student's *t*-test.

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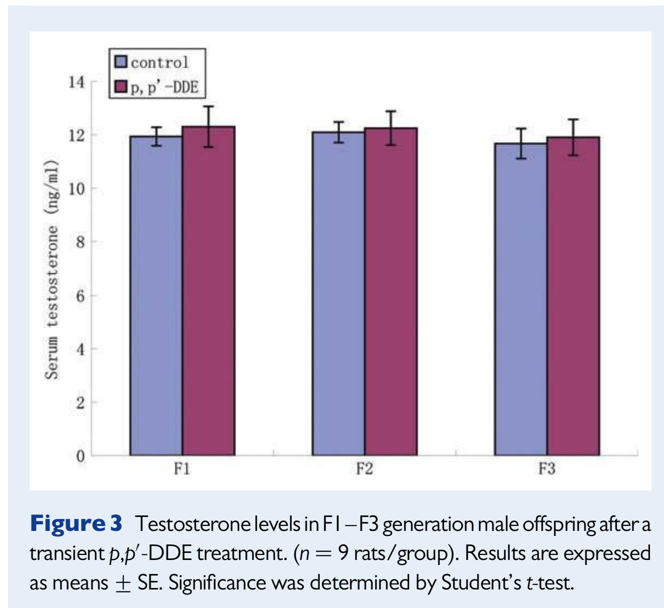

Figure 3 Testosterone levels in F1–F3 generation male offspring after a transient p,p′-DDE treatment. ($n = 9$ rats/group). Results are expressed as means ± SE. Significance was determined by Student's t-test.

Significant testicular apoptosis occurred at stages IX–XIV (Fig. 2C) but there was no effect at stages I–V and VI–VIII in p,p′-DDE generations (data not shown).

No significant difference in testosterone levels could be observed after a transient exposure to p,p′-DDE

To determine whether the fertility impairments were due to hormonal alterations, testosterone levels were analyzed. However, no significant differences in testosterone levels could be observed after the transient exposure to p,p′-DDE (Fig. 3).

H19 and Igf2 were up- and down-regulated in sperm of three generations after a transient exposure to p,p′-DDE

As shown in Fig. 4, the paternally expressed gene, *Igf2*, was down-regulated, while the maternally expressed gene, *H19*, was up-regulated in the sperm in the three generations after a transient exposure to p,p′-DDE.

Igf2 DMR2 was hypomethylated in sperm of three successive generations after a transient exposure to p,p′-DDE

To reveal the mechanisms underlying altered expression of imprinted genes, we analyzed the methylation level of 19 CpG sites at the *Igf2* DMR2. As shown in Fig. 5, the methylated percentage of methylation at the *Igf2* DMR2 was close to the theoretical values of 100% in the control. Significant hypomethylation at sites 1, 16 and 18 occurred for three successive generations after a transient treatment with p,p′-DDE.

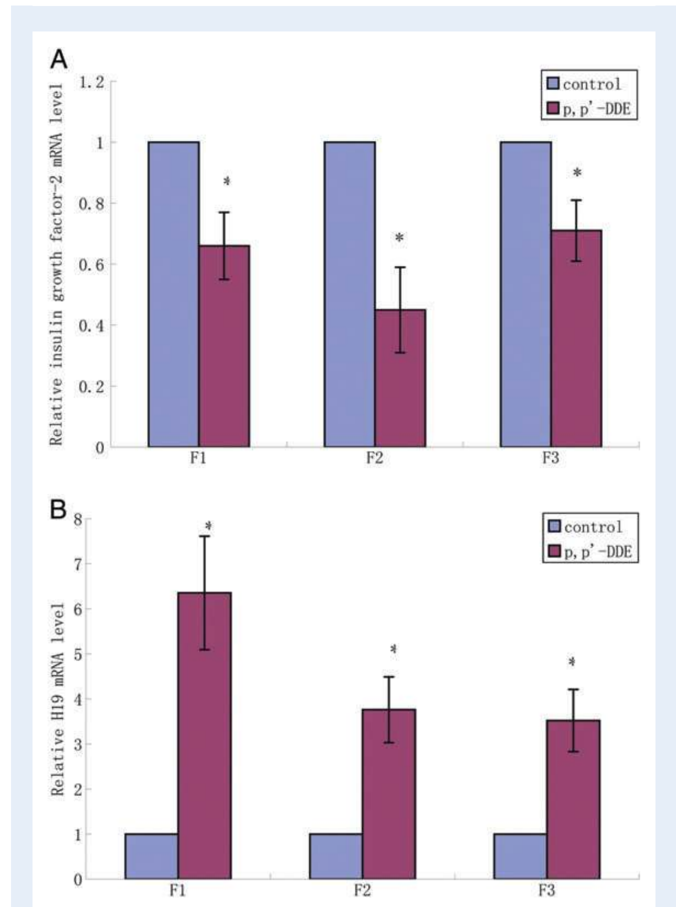

Figure 4 Relative mRNA expression of *Igf2* and *H19* in the sperm of F1–F3 generation male offspring after p,p′-DDE treatment. $^*P < 0.05$ versus control. Results are expressed as means ± SE ($n = 6$ rats/group). $^*P < 0.05$. Significance was determined by Student's t-test.

No difference in global methylation was found after a transient p,p′-DDE administration

In order to reveal whether the observed hypomethylation was locus specific, global 5-^{m}C was analyzed in the present study. Figure 6 showed that the 5-^{m}C per cent was 3–4% in the sperm of F1–F3 male offspring in both the treated and control groups. No significant differences could be observed in all the tested generations after the transient exposure to p,p′-DDE.

Discussion

Growing evidence suggests that exposure to an abnormal prenatal environment could cause chronic diseases including obesity and diabetes (Ding *et al.*, 2012; Skinner *et al.*, 2013a,b). Recently, a study has suggested that a detrimental fetal environment may also impair male fertility transgenerationally (Skinner *et al.*, 2010). In our study, p,p′-DDE could reduce transgenerational male fertility, with the effect being transmitted through the male germline and associated with altered expression of *Igf2* and *H19* caused by *Igf2* DMR2 hypomethylation.

p,p′-DDE, a representative endocrine disruptor, can interfere with the normal binding of androgen with its receptor (Kelce *et al.*, 1995).

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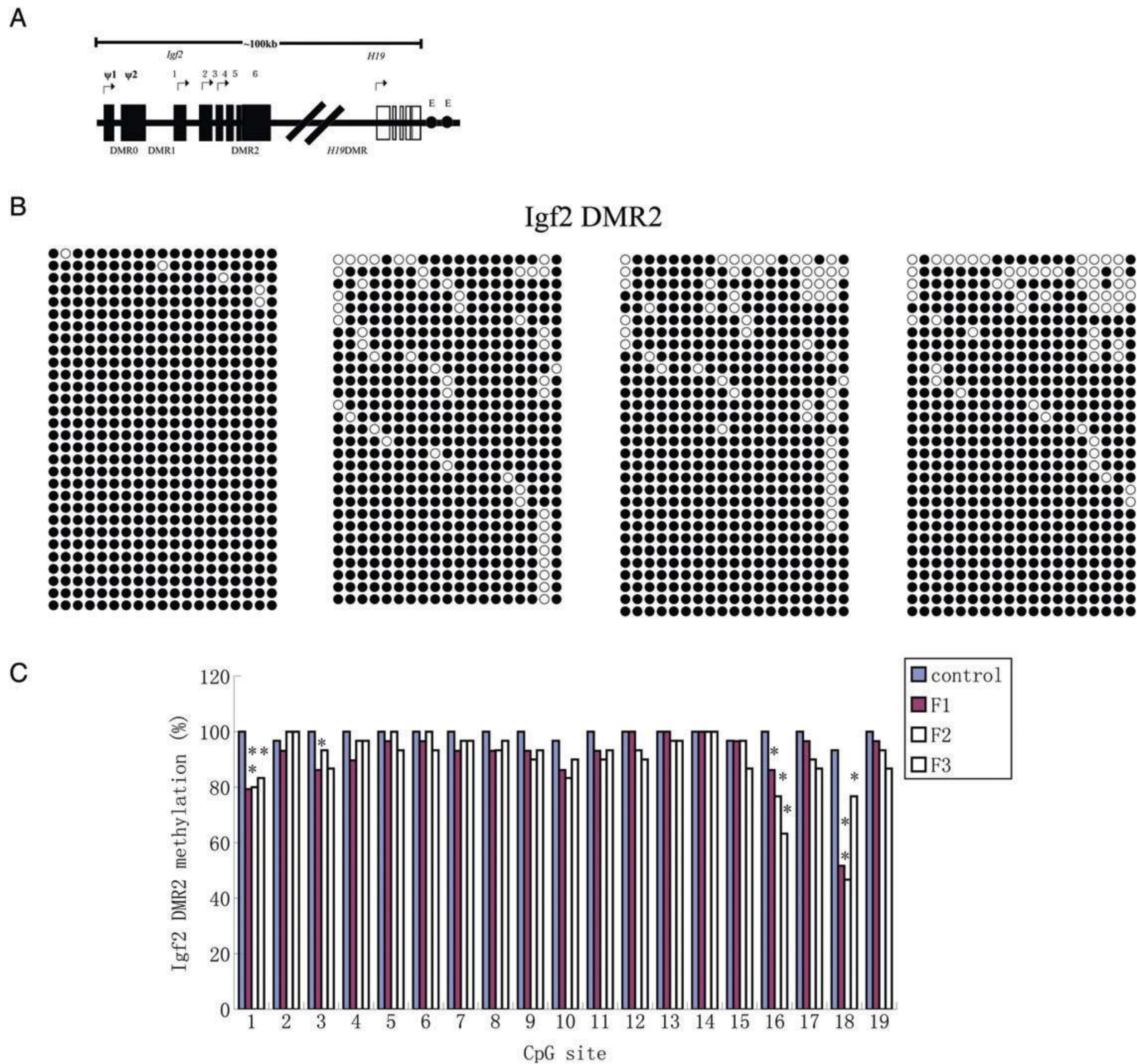

Figure 5 Methylation analysis of *Igf2* DMR2 by bisulfite sequencing. (**A**) Schematic representation of *Igf2*–*H19* DMR, indicating the location of the four DMRs known to contribute to *Igf2* imprinting. Exons are shown as black (Ψ1 and Ψ2) and white for *Igf2* and *H19* gene, respectively. Enhancers (E) are indicated as black circles. (**B**) The methylation status of individual DNA strands of *Igf2* DMR2 containing 19 CpG sites. A total of 30 clones per group were sequenced. Each line represents the sequence of a single clone. CpG sites are shown as blank (unmethylated) or filled (methylated) circles. (**C**) The total methylation ratio of *Igf2* DMR2 in each CpG site. Ten clones each from the three control and *p,p*′-DDE-treated groups were sequenced. For individual CpG sites, the percentage of methylated CpG sites out of the total number of CpG sites assessed for that site was determined. *$P < 0.05$ versus control. Significance was determined by χ^2 test ($n = 3$ rats/group).

Although we did not measure the actual burden of *p,p*′-DDE in dam and pups, You *et al* (1998) reported that the levels of *p,p*′-DDE in exposed fetal and feeding rats were comparable with that in human plasma from highly polluted regions, such as Mexico (López-Carrillo *et al*., 1996) or South Florida (Barquet *et al*., 1981). The exposure period was at a critical time when different testicular cell types were required to migrate and proliferate to form the seminiferous cord (Levine *et al*, 2000), and when imprinting is erased and reestablished in the germ cells (Davis *et al*., 2000).

A transgenerational effect is transmitted for a minimum of three generations through the germline, as there is no direct exposure of the F3 generation. In the present study, the sperm number and motility declined for three generations after a transient exposure to the endocrine disruptor *p,p*′-DDE. The outcross experiment, showing no phenotype in C♂-DDE♀, is consistent with Anway *et al*.'s study (2006), which indicated that male germline produced the transgenerational phenotype induced by the endocrine disruptor vinclozolin. In our study, an increasing degree of infertility in males occurred, with 3 and 4 out of 13 and 20

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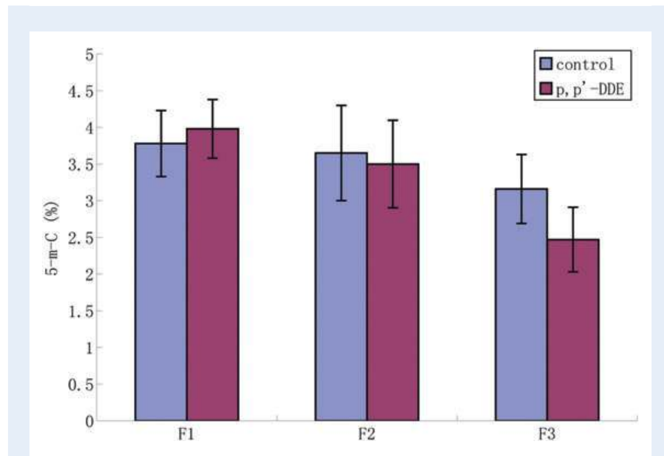

Figure 6 Global 5-methy-cytosine levels in sperm of F1–F3 generation male offspring after p,p′-DDE treatment. Results are expressed as means ± SE ($n = 4$ rats/group).

mice in the male-exposed F3 generation of DDE♂-C♀ and DDE♂-DDE♀, showing infertility compared with zero in the DDE F1 generation. Interestingly, the incidence of testis disease has increased in the 30 years after prenatal exposures to DDT (Cohn *et al.*, 2010). The most recent study has indicated that 50% of the F3 generation have developed obesity and testis disease after a transient exposure to DDT in F0 generation (Skinner *et al.*, 2013a,b). All of these studies suggest that germline-mediated detrimental effects caused by *p,p*′-DDE may be hidden for a long time before they are revealed.

Testosterone, which regulates germ cell differentiation through the somatic cells, plays a critical role in normal spermatogenesis. However, no significant differences in testosterone levels could be observed among the three generations after *p,p*′-DDE or no treatment. One possible explanation is that the function of the Leydig cells are not to be impaired in the absence of functional androgen receptors in the embryonic period (O'Shaughnessy *et al.*, 2002; Mu *et al.*, 2006). Hence, the transgenerational male reproductive toxicity caused by *p,p*′-DDE is not due to direct hormone alteration.

Although transgenerational inheritance of environmental effects is somewhat controversial (Heard and Martienssen, 2014), many studies indicated that germline reprogramming can play a critical role in the phenotype in the F3 generation (Skinner and Guerrero-Bosagna, 2009; Carone *et al.*, 2010; Skinner *et al.*, 2013a,b). An epigenetic defect could be retained and transmitted to further generations if there is an error in germline reprogramming; however, the mechanism involved is not clear (Youngson and Whitelaw, 2008). Studies have indicated that numerous loci are inclined to resist reprogramming at the time of demethylation, and these may be the prime candidates for transgenerational inheritance (Hackett *et al.*, 2012; Seisenberger *et al.*, 2012).

Although small RNA molecules and histone modifications are alternative mechanisms to be investigated in the transgenerational phenotypes (Ostermeier *et al.*, 2004; Aiken and Ozanne, 2014), more attention has been paid to DNA methylation (Reik *et al.*, 2001). In somatic cells, methylated imprinted genes are potential epigenetic targets for inactivation or over-expression due to their vulnerable functional haploid state (Murphy and Jirtle, 2000). Alterations in the sperm epigenome at DNA methylation sites can be used as biomarkers for transgenerational disease, while genome-wide analysis is being developed (Guerrero-Bosagna *et al.*, 2012; Skinner *et al.*, 2013a,b). Methylation patterns at the *Igf2/H19* imprinting control region have been found to be statistically significantly associated with sperm concentrations and motility (Poplinski *et al.*, 2010), as one of the critical epigenetic mechanisms currently known to affect male fertility. A minimal change of $<10\%$ methylation at the *Igf2/H19* ICR might have a profound effect on sperm quality and male fertility (Marques *et al.*, 2008; Boissonnas *et al.*, 2010; Marques *et al.*, 2010). The allelic expression of the *IGF2/H19* genes is regulated by a total of four *Igf2–H19* DMRs (Feil *et al.*, 1994; Lopes *et al.*, 2003). Since the *Igf2* DMR2 is the most labile region (Lopes *et al.*, 2003), it was chosen as the target domain in the present study. We found that *Igf2* DMR2 hypomethylation continued for three generations after *p,p*′-DDE treatment. The methylation changes present in the sperm might be inherited by the embryo, causing perturbations in the expression of *Igf2* and *H19* in the offspring (Doshi *et al.*, 2013), which might be the possible reason for the fertility loss induced by *p,p*′-DDE.

DNA methylation usually silences transcription. However, methylation is required for expression in many genes (Larsen *et al.*, 1992) including *Igf2*. DMR2 methylation plays a critical role in *Igf2* gene transcription, *and* a deletion of DMR2 could reduce *Igf2* mRNA levels (Murrell *et al.*, 2001). Although the mechanism is still unclear, Jones (1999) has hypothesized that transcription initiation from a CpG island could alter the chromatin structure to be a proper substrate for *de novo* methylation. Decreased *Igf2* and concurrent increased *H19* mRNA level indicate an up-regulation of the maternal allele and down-regulation of the paternal allele. Combined with the IGF-I receptor, Igf2 can induce the differentiation of spermatogonia into primary spermatocytes, playing a critical role in sperm cell survival and proliferation (Nakayama *et al.*, 1999; Burns and Hassan, 2001). Once Igf2 is down-regulated, the whole spermatogenesis process might be arrested at spermatogonial stage without further differentiation. Apoptosis of spermatogonia and spermatocytes could be induced without any proliferation, which might be the reason for the reduced sperm number. The apoptosis results also indicated that significant testicular apoptosis occurred at stages IX–XIV when early spermatocytes start to develop. Interestingly, vinclozolin (Stouder and Paoloni-Giacobino, 2010) was demonstrated to induce the same stage of spermatogenic cell apoptosis and imprinting alterations. Future studies of colocalization with specific marker proteins will be helpful to identify the cell types which are most sensitive to such factors.

Hypomethylation of the *Igf2* DMR2 could also result from a global decrease in methylation. To determine whether the observed hypomethylation was global or locus specific, the 5-^{m}C was analyzed in the present study. However, no significant difference in 5-^{m}C could be found, suggesting that the *Igf2* DMR2 hypomethylation was indeed locus specific in this study. Contrary to findings from our study, prenatal exposure to lower Alu methylation at birth has been associated with *p,p*′-DDE (Huen *et al.*, 2014). Rusiecki *et al.* (2008) and Kim *et al.* (2010) also found that Alu hypomethylation was associated with exposure to PCBs, DDT and POPs in adults. Epigenetic modifications are tissue specific and will vary between species, tissues and cells. In our study, sperms were studied in contrast with blood cells with varying levels of methylation in above epidemiology studies (Adalsteinsson *et al.*, 2012). The composition of blood cell could vary with environmental exposure (Peltier *et al.*, 2012). Thus, it is still unknown whether the Alu hypomethylation in the above studies is due to direct environmental exposure or due to confusion caused by cell type changes. Additionally, those studies have

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studied the Alu repeats, which were shown to be not correlated with the global genomic methylation content in non-cancer cells in a recent study (Price et al., 2012).

In the present study, *Igf2* DMR2 hypomethylation was analyzed to play a critical role in the transgenerational impairment of male fertility after a transient exposure to *p,p′*-DDE. Not only *Igf2* and *H19*, but also other imprinted genes (e.g. *Peg3*, *snrpn* and *Rasgrf1*) could be involved. Hence, further investigation is necessary to focus on the genome-wide epigenetic effects and a more detailed semen quality analysis including sperm morphology assessment is necessary.

In conclusion, impaired male fertility with epigenetic alterations is transgenerationally inherited after a transient exposure to *p,p′*-DDE, which raises etiological concerns in the investigation of related diseases.

Authors' roles

Y.S. and N.X.W. designed and performed the experiments. S.W. and M.G. collected the data. P.S. and J.L. analyzed and interpreted the data. Y.S. prepared the article. Y.F.T. and K.L. revised the article.

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Conflict of interest

No conflicts of interest are declared.

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