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ORIGINAL ARTICLE

Male Reproduction

Chronic exposure to ethanol in male mice may be associated with hearing loss in offspring

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Although paternal ethanol (EtOH) abuse has been shown to affect the growth and behavior of offspring, the exact molecular and mechanistic basis remains largely unclear. Methylation alterations in imprinted genes may be related to well-documented teratogenic effects of ethanol. Here we show that chronic paternal ethanol exposure increases the susceptibility to abnormal behavior in offspring through male game epigenetic alteration. In our study, different doses of ethanol (0, 1.1, 3.3 g kg^{-1}) were administered intra-gastrically to male mice and decreased sperm motility was found in the highest ethanol-exposed group compared with the controls. Data also showed a dose-dependent increase in deaf mice of the paternally ethanol-exposed groups. The methylation of *H19*, *Peg3*, *Ndn* and *Snrpn* was assessed in paternal spermatozoa and in the cerebral cortices of deaf mice. EtOH affected methylation of *Peg3* (CpG 3, 7 and 9) in paternal spermatozoa and in the cerebral cortices of deaf mice, but the level of mRNA expression did not change, suggesting that other gene regulation may be involved in these processes. Overall, chronic paternal ethanol exposure could alter the methylation of imprinted genes in sire spermatozoa that could also be passed on to offspring, giving rise to developmental disorders. Our results provide possible epigenetic evidence for a paternal ethanol exposure contribution to Fetal Alcohol Syndrome (FAS).

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INTRODUCTION

Increasing evidence suggests that certain acquired characteristics can be passed on to the next generation.[1] The majority of studies of trans-generational environmental inheritance described are associated with maternal effects. In recent studies, emerging evidence has supported the occurrence of paternal effects in their offspring. In addition, unlike the complexity of the maternal effect, fathers contribute directly little more than spermatozoa to offspring. Few studies have been performed to examine the paternal effects on the offspring.[1]

The teratogenic effects of ethanol exposure have been well-documented. Increasing evidence suggests that paternal alcohol consumption affects offspring development, especially neuro-developmental disorders. In a mouse model, paternal ethanol exposure is associated with smaller litters and an increased male/female offspring ratio.[2,3] Male mice exposed to ethanol have been associated with growth retardation and behavioral abnormalities in offspring.[4,5] In a clinical study, a correlation was observed between chronic paternal alcohol consumption and impaired cognitive skills and hyperactivity in the children of these fathers.[6] In other studies, significant decreases in infant birth weight and septal defects have also been correlated with paternal alcohol consumption.[7,8]

Sensory deficits can play a major role in neuro-development disorders. Some reports have shown that prenatal alcohol exposure can cause hearing loss and abnormal cortical processing of auditory information.[9,10] In human studies, hearing disorders have been found in children with the fetal alcohol syndrome.[11,12] However, none of the current studies have reported hearing disorders in the offspring of male mice exposed to ethanol. In our previous study, a specific circling behavior was observed in the offspring of the paternally ethanol-exposed group. During the course of our analysis of the circling behavior, we observed that some animals may also have an insensitive hearing behavior. Thus, our particular interest concerns are hearing disorders in this study. As of yet, the exact mechanistic basis of how paternal ethanol exposure contributes to offspring defects remains largely unclear.

Evidence suggests that regulation of epigenetic processes is maintained throughout spermatogenesis.[13] It not only ensures proper sperm function, but also embryonic development. Epigenetic regulation is known to change with the environment, and the mechanism of epigenetic inheritance has been associated with the paternal effect on offspring. Imprinted genes, which are regulated by DNA methylation at differentially-methylated regions (DMRs), have been linked to human growth and developmental disorders.[14] Ethanol is an excellent candidate for perturbing genomic imprinting by inhibiting methionine synthase and methionine adenosyl-transferase. The evidence suggests that ethanol can regulate expression of imprinted genes by reducing

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methyl-transferase activity, which affects epigenetic reprogramming of DMRs.[15] Furthermore, ethanol has been shown to alter DNA methylation in imprinted gene DMRs in paternal spermatozoa[15] and mouse offspring.[16] Previously, we have identified some altered CpG sites of imprinted genes, which were induced by paternal ethanol exposure and that could pass to the offspring, thereby resulting in neuro-developmental disorders;[17] Besides that, there is no other evidence for aberrant methylation of imprinted genes being passed on to offspring via spermatozoa and leading to the developmental disorders.

In this study, we investigated the paternal ethanol association in mice by treating males with different doses of ethanol (intra-gastric administration of 0, 1.1, 3.3 g kg^{-1}). Sperm function in paternal mice and the hearing-related behavioral changes were assessed in their offspring. Four imprinted genes (*H19*, *Peg3*, *Ndn* and *Snrpn*) were evaluated in paternal spermatozoa and cerebral cortices of these offspring to explain chronic ethanol-induced paternal germ-line epigenetic effects on offspring development.

MATERIALS AND METHODS

Animals

Kunming (KM) mice (Swiss albino mice origin) were purchased from the Animal Centre of the Chinese Academy of Sciences. The mice were housed and maintained at 22 ± 0.5°C, and a 12:12 h light/dark cycle with free access to food and water. All experiments were conducted in strict accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The 103 male KM mice aged 5–6 weeks (weight, 20–25 g) were randomly divided into the following three groups: exposure to 3.3 g kg^{-1} ethanol (n = 45), exposure to 1.1 g kg^{-1} ethanol (n = 39), and exposure to distilled water 0 g kg^{-1} (n = 39). The mice were then intubated intra-gastrically and given ethanol or water every 2 days for 4 weeks. All efforts were made to minimize animal suffering.

After 1 month of treatment, males were naturally mated with untreated females within 2–3 days to breed the F1 generation. The presence of a copulation plug in female mice was presumed to indicate mating, and then male mice were killed and the spermatozoa were collected from the cauda epididymidis for analysis and DNA extraction. Eight weeks after the F1 birth, behavioral and auditory tests were conducted to assess hearing.

Analysis of sperm motility

Sperm motility was analyzed by using a computer-assisted semen analysis (CASA) system, the Hamilton Thorne Research Motility Analyzer (HTM-IVOS with software version 12.3, Beverly, MA, USA). Spermatozoa from the cauda epididymidis were washed and incubated in HTF medium (Quinn's Advantage Fertilization, Trumbull, CT, USA). The tissue was minced with scissors and incubated at 37°C and 5% (v/v) CO_2 for 5 min to allow the sperm cells to swim out. Suspensions of spermatozoa were loaded into flat 100 μm deep microslides (HTR1099, VitroCom Inc., Mt. Lks. NJ, USA) for computer-assisted sperm analysis. For each sample, 10 randomly selected fields containing >200 moving sperm cell tracks were examined at 60 Hz. The kinetic parameters of sperm were monitored as follows: the percentage of motile spermatozoa (Motile), the percentage of spermatozoa with progressive movement (Progressive), curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), amplitude of lateral head displacement (ALH), and linearity% (LIN, VSL/VCL × 100%)

Auditory brainstem responses recording

The auditory brainstem response (ABR) test has been described as a method for assessing auditory nerve and brainstem lesions. In our study, F1 mice from each experimental group were tested for the auricle reflex. Subsequently, the hearing insensitivity of mice would be confirmed by ABR testing. Animals were anaesthetized with a solution of ketamine and xylazine (40 mg kg^{-1} and 8 mg kg^{-1}, i.p., respectively). The body temperature was maintained at approximately 37 ± 1°C by a thermostatic heating pad. Evoked responses were collected from three subdermal electrodes positioned at the vertex and the mastoid regions (an earth electrode was placed on the vertex in the scalp midline, a reference electrode was placed in the left mastoid area, and the active one was placed in the right mastoid area). Recordings were obtained from the TDT system III and software (Tucker-Davis Technologies, Alachua, FL, USA). Responses to click stimuli and to tone bursts at 1, 2, 4, 8, 16 and 32 kHz with a duration of 10 ms were recorded and the threshold was defined as the lowest sound level at which a distinct ABR waveform could be recognized. The measurement was continued with reductions of 10 dB from the 100 dB hearing level.

DNA extraction and sodium bisulfite conversion

DNA from paternal spermatozoa and from the offsprings' cerebral cortices was extracted by using a QIAamp DNA Min kit (Qiagen, Valencia, CA, USA). The concentration and purity of DNA were determined using absorbance at 260 and 280 nm in a NanoDrop TM 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Bisulfite conversion of DNA was performed by using the EZ DNA Methylation Kit™ (Zymo Research, Orange, Irvine, CA, USA). Converted DNA was resuspended in 10 μl elution buffer and stored at −80°C before analysis.

Quantitative MassARRAY analysis of gene methylation status

The Sequenom MassARRAY platform (San Diego, USA) was used to perform the quantitative methylation analysis of imprinted genes. This system, which combines base-specific enzymatic cleavage with matrix-associated laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, is a highly accurate, sensitive and high-throughput method for the quantitative analysis of DNA methylation at CpG sites.[18] DNA methylation at selected imprinted gene DMRs of four imprinted genes were quantified with the Sequenom MassARRAY EpiTYPER analyzer. The robustness of this approach for quantifying methylated/unmethylated DNA has been demonstrated by Sequenom. Primers used in this study were designed by using Methprimer (http://epidesigner.com). The primer sequences and methods of the imprinted genes are mentioned in our previous study.[17] The spectra and the methylation values of MALDI-TOF mass spectrometry (Sequenom) were collected and analyzed by using Epityper software (version 1.0; Sequenom). The DNA methylation assays were performed in triplicate. Inapplicable readings and their corresponding sites were eliminated from analysis. The average methylation was calculated as the mean value of the CpGs methylation rate and expressed as a relative amount of methylation.

RNA extraction and quantitative RT-PCR

Total RNA was extracted from the offspring's cerebral cortical samples with Trizol Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. RNA was reverse-transcribed by using a PrimeScript RT reagent Kit with gDNA Eraser (Perfect Real Time, TaKaRa, Otsu, Shiga, Japan) and the integrity of synthesized cDNA was confirmed by using glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) as the endogenous control. Quantitative RT-PCR was performed in an ABI 7900 real-time PCR system with SYBR Premix Ex Taq (Perfect Real-Time, TaKaRa). Reactions were performed in triplicate and analyzed by using an ABI 7900 Sequence

Detection System (Applied Biosystems). Primer sequences used in the QRT-PCR analysis are listed in our previous study.[17]

Statistical analysis

Results were expressed as the mean ± s.e.m. Statistical analysis was performed with SPSS statistical software (version 13.0, IBM Corporation, Armonk, NY, USA). Differences between the control and the ethanol treatment groups were analyzed by using one-way analysis of variance (ANOVA) and the Bonferroni *post hoc* test. The level of significance was set at $P < 0.05$.

RESULTS

Male mice were treated with different doses of ethanol, and the sperm parameters were evaluated by CASA analysis 1 month later. Data revealed that the sperm motility was decreased in the highest ethanol-exposed groups compared with the control group. As shown in **Figure 1**, the 3.3 g kg^{-1} ethanol-exposed mice had significantly decreased sperm motility compared with that of the control group (F (2,48) = 4.023; $P = 0.024$). Compared with the control animals, the progressive sperm motility of mice exposed to 3.3 g kg^{-1} ethanol was also decreased, but the difference was not statistically significant. In addition, there were also no statistically significant differences in VAP, VCL and VSL of sperm between the control and ethanol-exposed groups (**Figure 1**).

Hearing function was also assessed in the F1 generation of paternally-exposed ethanol and controls. Lack of a Preyer reflex (ear flick in response to noise) was observed in a small percentage of the offspring of the paternal ethanol-exposed group, which suggests a severe hearing impairment. To test the hearing sensitivity of these mice further, we performed ABR threshold measurements. The results showed that there was no ABR response at a 100 dB sound pressure level (SPL) at any frequencies tested compared with the controls, suggesting deafness (**Figure 2**). We also found that the deafness and circling behavior increased with increasing ethanol (**Table 1**). This phenotype was not been found in the control group.

To study possible trans-generational effects, the methylation in DMRs of four imprinted genes (*H19*, *Peg3*, *Ndn* and *Snrpn*) was analyzed in paternal spermatozoa and cerebral cortices of deaf mice. For *H19* DMRs in paternal sperm cells, the group exposed to 3.3 g kg^{-1} ethanol had less methylation (F (2,28) =3.950; $P = 0.030$) than the control group. In *Peg3* gene of spermatozoa, the males with the highest ethanol exposure had a 7% (F (2,24) =5.732; $P = 0.031$) higher mean total methylation than controls (**Figure 3**). We also assessed every CpG site in these genes. Significant differences were observed in CpG3-CpG11 sites in paternal spermatozoa of *Peg3* (**Figure 4**). As shown in **Figure 5**, the same positions were evaluated in deaf mice of paternal ethanol exposure. A 6%–7% more methylation of CpG3 (F (2,15) =5.515; $P = 0.016$), CpG7 (F (2,15) =5.464; $P = 0.016$), and CpG9 sites (F (2,15) =5.274; $P = 0.018$) in *Peg3* DMRs were observed in the cerebral cortices of deaf offspring of the highest paternal exposure group compared with controls. However, by comparing the level of mRNA expression of *Peg3* in cerebral cortices among deaf groups and controls, no statistically significant differences were observed. In addition, no statistically significant differences in the mean relative methylation for *Ndn* and *Snrpn* were observed between the ethanol treatment and control groups in paternal spermatozoa and in cerebral cortices of deaf mice (**Figure 3**).

DISCUSSION

We have shown that ethanol exposure in male mice may affect sperm parameters and gene methylation and that the altered methylation may be transmitted to offspring resulting in developmental disorders. Our results provide possible epigenetic evidence that paternal ethanol exposure contributes to FAS.

Alcohol abuse has detrimental effects on male fertility and embryonic development. Many studies have shown that chronic ethanol exposure affects testosterone levels, disturbs the blood-testis barrier, and perturbs spermatogenesis.[19,20] Other reports have also suggested that alcohol consumption is associated with a reduction in sperm motility and increased morphological abnormalities.[21] However, the molecular mechanisms of these effects are still under investigation. In this study, different doses of ethanol (0, 1.1, and 3.3 g kg^{-1}, intra-gastrically) were administered to male KM mice, and the effects on their sperm function were assessed. These data showed that the velocity characteristics (VCL, VSL and VAP) did not differ among the three groups. We found significantly decreased sperm motility in the 3.3 g kg^{-1} ethanol-exposed mice compared with the control group, which is consistent with a previous study in which alcohol consumption in men was reported to be related to decreased sperm motility.[22] However, there was no difference in sperm motility between male mice

Table 1: The percentage of circling behaviour and deafness in mice

Ethanol (g kg^{-1})	*Circling behaviour (%)*	*Deafness (%)*	*Circling and deafness (%)*
0	0	0	0
1.1	0.32	0.96	0
3.3	1.57	2.76	0.32

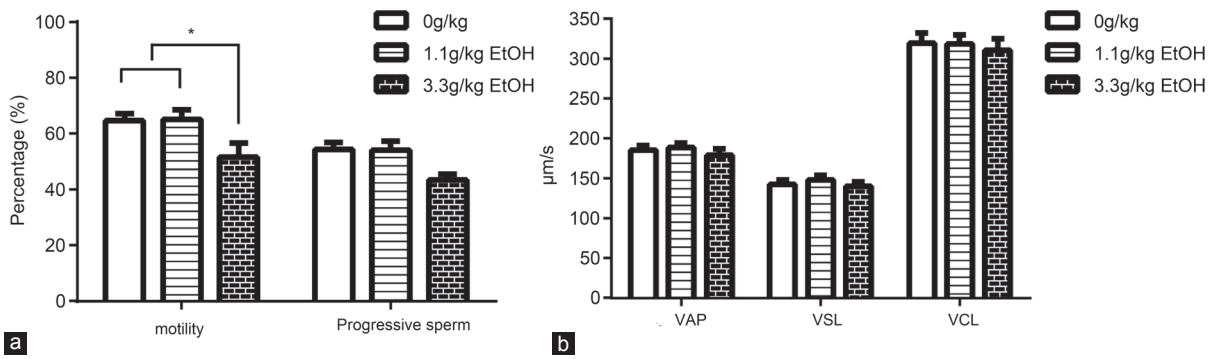

Figure 1: Effect of different doses of ethanol on sperm parameter values in F0 mice (ethanol, 1.1 g kg^{-1}, 3.3 g kg^{-1}; n = 15–18 per group). (**a**) Percentage sperm motility and progressive motility. (**b**) Curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP). The characteristic of parameters obtained by using the computer-assisted semen analysis (CASA) system. Data are mean ± s.e.m. *$P < 0.05$, compared with control group.

exposed to 1.1 g kg^{-1} ethanol and control mice. Although progressive sperm motility was reduced in males exposed to 3.3 g kg^{-1} ethanol, there was no statistical difference between the ethanol-exposed groups and control mice. However, other reports of paternal ethanol effects on progressive sperm motility are inconsistent.[21,23] The discrepancies in the effects of ethanol on reproductive parameters may in part, be due to different dosages and methods of administration.

Paternal ethanol exposure could impair male reproductive function and affect the offspring's development. Our study confirms the association between paternal ethanol exposure and hearing disorders in the offspring. This is the first demonstration that paternal ethanol exposure can cause a dose-dependent increased deafness in offspring, although other studies have focused on the mother's effects. In clinical and animal studies, hearing disorders have been found in the offspring of mice with prenatal ethanol exposure. Therefore, there are several mechanisms that may be responsible for the hearing disorder. For example, ethanol induces cell damage in the embryonic inner ear and impairs the auditory brainstem structures, thereby resulting in hearing disorders.[24,25] It has also been shown that altered epigenetic modifications induced by ethanol change the hypothalamic-pituitary-adrenal (HPA) axis activity in the mother and fetus.[26] Nevertheless, our study demonstrates an effect of ethanol on spermatozoa, and that altered information is transmitted to the offspring, leading to hearing disorders. In addition, the deafness in our study was accompanied by other abnormalities. We have previously observed a circling behavior in the offspring of paternal ethanol-exposed groups, and it is interesting to find here that this was dose-dependent. This finding is consistent with the fetal alcohol syndrome, which is a collection of physical and neuro-development impairments, such as cognitive and learning disabilities, auditory deficits, impaired balance skills.[27,28]

Proposed mechanisms to explain these underlying changes in behavior have included aberrant DNA methylation of specific

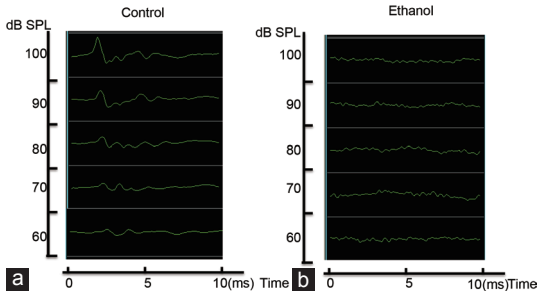

Figure 2: (**a**) The auditory brainstem response thresholds of controls. (**b**) The auditory brainstem response thresholds of deaf mice induced by paternal ethanol exposure.

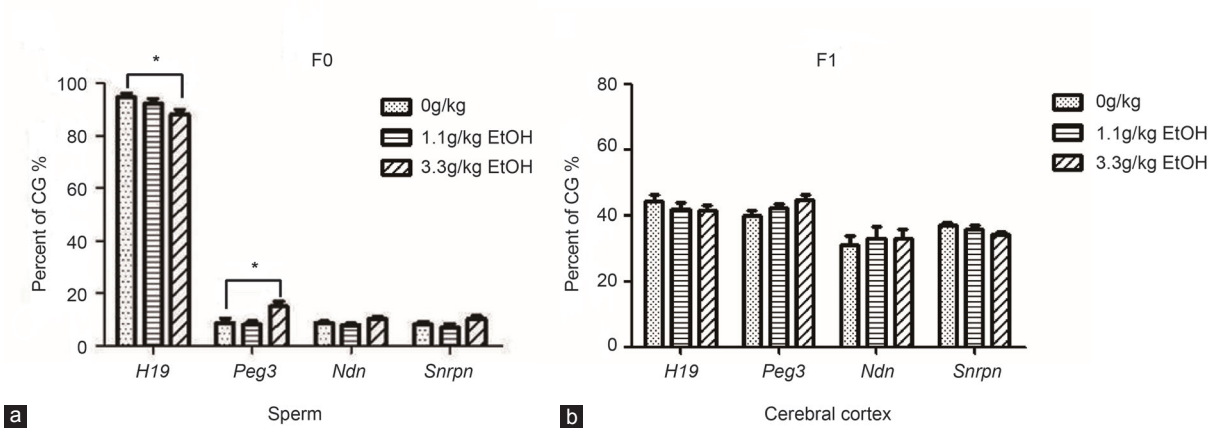

Figure 3: (**a**) Methylation status of four imprinted genes in paternal spermatozoa of control and ethanol-exposed mice. (**b**) Methylation of four imprinted genes in cerebral cortices of deaf mice and control. Results are mean ± s.e.m. *$P < 0.05$, compared with control group.

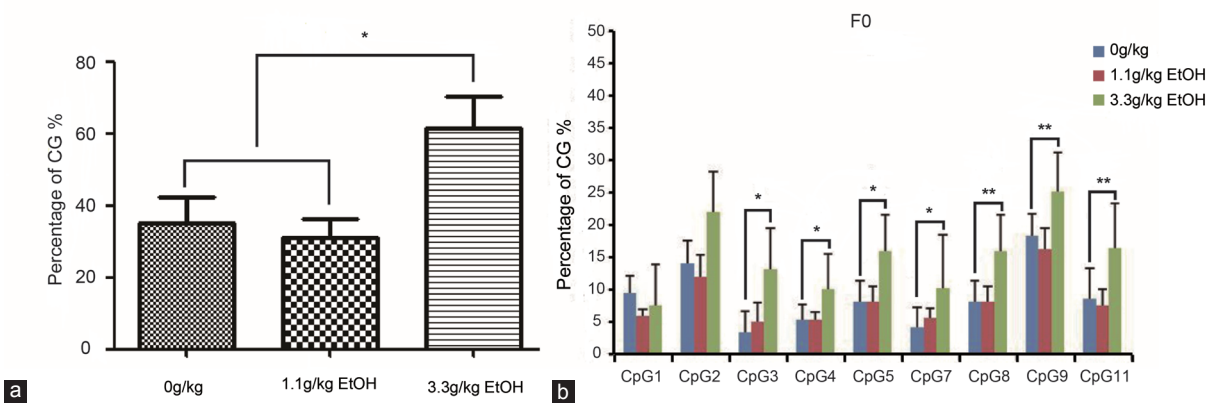

Figure 4: (**a**) Methylation status of *Peg3* gene in paternal spermatozoa of control and ethanol-treated mice. (**b**) Methylation of the CpG site of *Peg3*'s differentially-methylated region (DMR) in control and ethanol-treated groups. Results are mean ± s.e.m. of 10–12 mice. *$P < 0.05$; **$P < 0.01$, compared with control group.

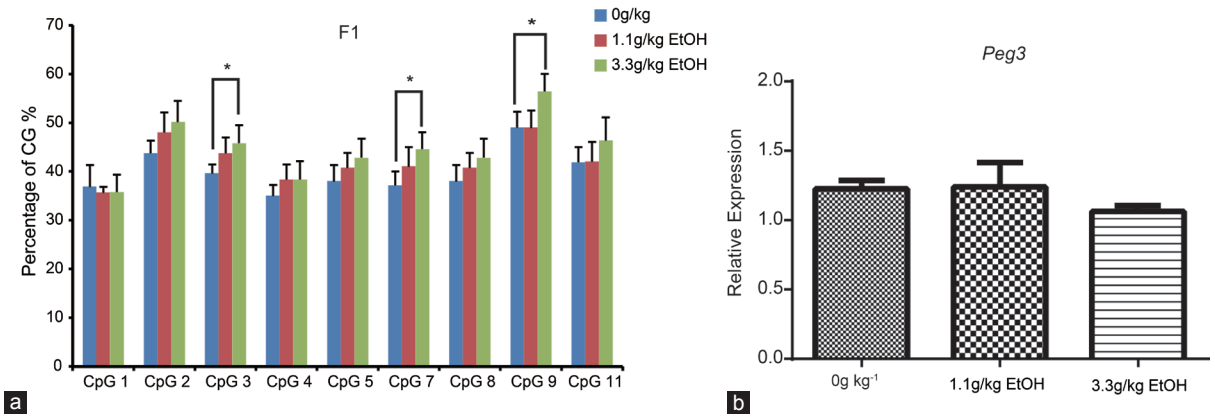

Figure 5: (**a**) Methylation of CpG site of *Peg3*'s differentially-methylated region (DMR) in cerebral cortices of ethanol-induced deaf mice and controls. (**b**) Level of mRNA expression of *Peg3* genes in cerebral cortices of deaf mice and controls. Results are mean ± s.e.m. **P* < 0.05, compared with control group.

genes by ethanol, which may lead to mental deficits and abnormal behavior. It has been found that genomic imprints are critical for the maintenance of fertility; therefore aberrant methylation in spermatogenesis could have a profound effect on both male fertility and embryonic development. Studies have shown that paternal ethanol exposure and the consequence of abnormalities in offspring may be caused by altered genomic imprinting mediated by changes in DNA methylation.[17] Previously, we have identified four imprinted genes (*H19*, *Peg3*, *Ndn* and *Snrpn*) that were closely related to fetal growth and neuro-development. The highest ethanol exposure altered the methylation and mRNA expression of *Peg3*, which was observed in mice with circling behavior but not in the controls. Therefore, our present study extends our previous findings, which also identified the methylation of these four imprinted genes in the effect of paternal ethanol exposure onto their offspring. Our data confirm a pattern of significantly increased methylation at the *Peg3* DMR in sperm DNA of male parents exposed to 3.3 g kg^{-1} ethanol. However, the same increased methylation at only three CpG sites (CpG 3, 7, and 9) in the *Peg3* DMR was found in the cerebral cortices of the deaf mice. *Peg3* (paternally expressed gene 3) encodes a Kruppel-type zinc finger-containing protein, which is known to interact with several key proteins for various cellular processes. For example, it can be involved in the p53-mediated apoptosis and can also can inhibit the Wnt signaling pathway, which may result in developmental disorders.[29,30] Together with previous work this indicates that altered methylation can also be transmitted from spermatozoa to offspring. In addition, the altered inherited methylation was not found in other genes. Furthermore, evidence suggests that even a single CpG sites may elicit a significant biological effect. However, no difference was observed in the mRNA expression of *Peg3* between the deaf mice and controls. *Peg3* involved in this mechanism of paternal ethanol exposure effect the deafness in the offspring. However, we assume that other genes are also associated with the regulation of the deafness, and these have yet to be identified.

In summary, we have discovered a unique dose-dependent deafness in the offspring of male mice exposed to ethanol. In addition, we also characterized the possible mechanism for the epigenetic inheritance. However, other candidate genes may be at play in these complex mechanisms. To explain the deafness phenomena completely, further studies will evaluate the functional gene families instead of single genes to build on our data.

AUTHOR CONTRIBUTIONS

FL carried out the animal studies and performed the statistical analysis and drafted the manuscript. LD carried out the molecular studies and participated in the statistical analysis. NJ, JZ, HJW, WHZ and GYH participated in its design and coordination. DM conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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