Influence of fetal Leydig cells on the development of adult Leydig cell population in rats

Dong-Mei SU1)*, Ying FENG2)*, Lin WANG3), Yi-Lun WU3), Ren-shan GE5) and Xue MA1)

1) Department of Pediatric Urology, West China Hospital, Sichuan University, Chengdu 610041, China
2) West China School of Basic Medical Sciences & Forensic Medicine, Sichuan University, Chengdu 610041, China
3) Key Laboratory of Obstetric, Gynecologic and Pediatric Diseases and Birth Defects of Ministry of Education, West China Second University Hospital, Sichuan University, Chengdu 610041, China
4) West China Hospital, West China School of Clinical Medicine, Sichuan University, Chengdu 610041, China
5) Center of Scientific Research, The Second Affiliated Hospital and Yuying Children’s Hospital of Wenzhou Medical University, Zhejiang 325027, China

Abstract. Leydig cells are the main endogenous testosterone synthesis cells in the body. Testosterone is an essential hormone in males that affects metabolism, emotion, and pubertal development. However, little is known about the development of Leydig cells and relationship between fetal Leydig cells (FLCs) and adult Leydig cells (ALCs). The aims of this study were to investigate the effect of (FLCs) on ALC development. Our study showed that FLCs in neonatal rat testis can be eliminated by 100 mg/kg ethane dimethane sulfonate (EDS) treatment without affecting the health of newborn rats. Immunohistological results showed that eliminating FLCs led to early re-generation of the ALC population (progenitor Leydig cells [PLCs] and ALCs) accompanied at first by increased and then by decreased serum testosterone, indicating that ALCs which appeared after neonatal EDS treatment were degenerated or had attenuated functions. Our results showed that FLCs were eliminated 4 days after EDS treatment, the ALC population regenerated by 21 days, and serum testosterone levels dramatically decreased at 56 days. Collectively, our results indicate that the ablation of FLCs in neonatal rat results in abnormal development of ALCs. Our study further indicates that abnormal development of Leydig cells in the fetal stage leads to steroid hormone disorders, such as testosterone deficiency, in the adult stage. Therefore, studies of Leydig cell development are important for understanding the pathogenesis of testosterone deficiency or pubertas praecox.

Key words: Adult Leydig cell, Ethane dimethane sulfonate, Fetal Leydig cells, Leydig cell, Rat, Testosterone

Journal of Reproduction and Development, Vol. 64, No 3, 2018

—Original Article—

Testosterone is an androgen steroid hormone that contributes to regulating sexual desire, erectile function, and carbohydrate, fat, and protein metabolism in men [1, 2]. Testosterone deficiency has been reported to play a key role in the pathology of numerous diseases, such as prostate cancer [3, 4], liver diseases [5], and cardiovascular diseases [6]. Testosterone deficiency is common among males aged 65 years and older [7]. Studies have shown that aging contributes to testosterone deficiency [8]. However, Szarvas et al. [9] found that male testosterone deficiency occurred independently of age and had high morbidity. The causes of testosterone deficiency are largely unknown. Therefore, identifying the factors determining the male testosterone level is very important for revealing the cause of testosterone deficiency.

Leydig cells are the main steroidogenic cells in the male testis. There are two types of Leydig cells, fetal Leydig cells (FLCs) and adult Leydig cells (ALCs), in the fetal and adult testis, respectively [10]. Morphologically, FLCs are 3β-hydroxysteroid dehydrogenase (3β-HSD)-positive, while ALCs are cytochrome P450 family 17 (CYP17)- and 17β-HSD3 (HSD17B3)-positive in both mouse and rat [11, 12], and thus they can be distinguished by 3β-HSD or 17β-HSD staining. Functionally, FLCs cannot synthesize testosterone independently because they lack HSD17B3, while ALCs synthesize testosterone from cholesterol [13]. However, with the help of fetal Sertoli cells, FLCs can convert androstenedione to testosterone as observed in mice [13, 14]. In rats, FLCs are found in the fetal testis, but gradually degenerate and are replaced with ALCs after birth [15]. According to Lording and de Kretser, FLCs began to decrease in postnatal life and reached a minimum at 14 days after birth in rat [16]. Recent studies indicated that FLCs also exist in the adult testis in mice [12]. However, ALCs are not derived from FLCs [17]. While the origin of FLCs remains unclear (reviewed by Q. Wen) [18], some evidence has shown that ALCs arise from stem Leydig cells (SLCs) through two intermediate cells, progenitor Leydig cells (PLCs) and immature Leydig cells (ILCs) [19]. SLCs can self-renew and differentiate into several cell lineages, including LCs, while SLCs are unable to secrete testosterone until they differentiate into other LCs. PLCs are spindle-shaped and luteinizing hormone receptor- and HSD3B1-positive, but weakly positive for HSD17B3.

Received: August 16, 2017
Accepted: February 9, 2018
Published online in J-STAGE: March 6, 2018
©2018 by the Society for Reproduction and Development
Correspondence: X Ma (e-mail: medmaxue@163.com), R-s Ge (e-mail: r_ge@yahoo.com)
* D-M Su and Y Feng contributed equally to this article.
This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License. (CC-BY-NC-ND 4.0: https://creativecommons.org/licenses/by-nc-nd/4.0/)
Animals, treatments, and sample collection

Radioimmunoassay (RIA) in the testis [25–27]. Finally, we evaluated the effect of FLCs on Leydig-cell-cytotoxic molecule, to specifically ablate Leydig cells with ethane dimethane sulfonate (EDS), a widely used specific degeneration of FLCs. In this study, we treated a neonatal rat model the development of ALCs, as the development of ALCs begins with increasing evidence has shown that fetal programming can influence adult testosterone levels [22–24]. Thus, we hypothesized that abnormal ALC development affects testosterone levels in adult males. Furthermore, we hypothesized that FLCs influence the development of ALCs, as the development of ALCs begins with degeneration of FLCs. In this study, we treated a neonatal rat model with ethane dimethane sulfonate (EDS), a widely used specific Leydig-cell-cytotoxic molecule, to specifically ablate Leydig cells in the testis [25–27]. Finally, we evaluated the effect of FLCs on the development and steroidogenic function of ALC-related cells.

Materials and Methods

Animals, treatments, and sample collection

All 3-day-old Sprague-Dawley male rats used in this study were purchased from the Laboratory Animal Research Center of Rockefeller University (New York, NY, USA). All animal procedures were approved by the Institutional Animal Care and Use Committee of Sichuan University. All experiments were performed according to approved guidelines.

Rats with similar weights were randomized into experimental and control groups. Experimental groups were injected with 75, 100, and 125 mg/kg of EDS (kindly provided by Dr. RS Ge, Population Council & Rockefeller University), respectively. EDS was dissolved in dimethyl sulfoxide (DMSO) solution (DMSO: PBS, 1:3, v/v). The control group was injected with the same volume of DMSO solution. Each rat was intraperitoneally injected once with 50 μl of EDS or DMSO solution. The rat was held by the left hand to ensure that the rat’s abdomen faced upward, the syringe was held in the right hand, and injection was conducted at a 45° angle into the abdominal cavity then the needle angle was decreased to inject prevent the needle from damaging the abdominal viscerca. Blood samples and the testis, seminal vesicle, and brain were collected at specific times. Blood samples were collected and centrifuged to collect the sera, which were stored at −20°C until serum testosterone measurement. Next, the rats were sacrificed, and one testicle from each rat was harvested and frozen for subsequent immunohistochemical and immunofluorescence staining, while the other testicle as well as accessory sex gland organs and the brain of each rat were collected and stored at −80°C until analysis of mRNA and protein levels.

Radioimmunoassay (RIA)

Serum testosterone concentrations were measured with a testosterone radioimmunoassay (RIA) kit (Immunodiagnostic Systems, Boldon, UK) according to the manufacturer’s instructions using testosterone (Sigma Chemical, St. Louis, MO, USA) as a standard.

Immunohistochemistry

Staining of Leydig cell markers was carried out as previously described [24]. Briefly, 3β-HSD, 17β-HSD, or HSD11B1 staining solution containing DHEA (Steraloids, Wilton, NH, USA), nitroblue-tetrazolium (NBT; Sigma Chemical) and β-nicotinamide adenine nucleotide (NAD+; Sigma Chemical) was dissolved in PBS. Frozen rat testis sections (10 μm) were incubated with 3β HSD, 17β HSD, or HSD11B staining solution for 15 min at 25°C, washed three times with ddH2O, mounted with 50% glycerol solution (v/v), and observed by microscopy (DMD108, Leica, Wetzlar, Germany).

The frozen left testis samples of three 56-day-old rats were cut into serial sections. Three visual fields of the testicular interstitium were randomly chosen and photos were acquired. HSD11B-positive cells and total cells in the picture were counted. The same cell counting was conducted for every 5 sections throughout the whole testis. The total HSD11B-positive cell number and total cell number in all photos were recorded for further data processing.

Immunofluorescence Staining

Frozen sample sections were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 at room temperature for 20 min. After serum blocking, the sections were incubated with anti-rat HSD11B1 antibody (rabbit monoclonal, 1:100, DakoCytomation, Glostrup, Denmark) at 37°C for 45 min and washed with PBS-Tween 20. The sections were incubated with secondary antibody for 30 min at room temperature in the dark, washed with PBS-Tween 20, and mounted. Data were acquired using a fluorescence microscope (Leica).

Reverse transcription-polymerase chain reaction (RT-PCR) and real-time quantitative PCR (qPCR)

Total RNA was extracted from the samples using Trizol reagent (Molecular Research Center, Cincinnati, OH, USA). cDNA was synthesized from the isolated total RNA by RT-PCR with M-MLV reverse transcriptase (Promega, Madison, WI, USA, USA). Specific mRNA levels were measured by qPCR using SYBR reagent after RT-PCR. qPCR was carried out in a 20-μl volume system using SYBR Green PCR Core Reagents (New England Biolabs, Ipswich, MA, USA) according to standard qPCR protocols and performed on Light Cycler 2.0 (Roche Diagnostics, Basel, Switzerland). The primers used are listed in Table 1.

Western blotting analysis

After being triturated within liquid nitrogen, tissues were sonicated in radio immunoprecipitation assay buffer (RIPA) with protease inhibitor cocktail (Sigma-Aldrich) and 2 mM PMSF, and then centrifuged to collect the supernatant. The protein concentration was measured with a BCA protein Assay kit (CWBIO, Beijing, China). The samples were mixed with 5X SDS loading buffer. Approximately 100 μg of each protein sample was used for detection. The proteins were separated by SDS-PAGE and then electroblotted onto polyvinylidene difluoride membranes (Amersham Biosciences/GE Healthcare Little Chalfont, UK). The membranes were blocked in PBS containing 5% non-fat milk and 0.1% Tween 20 and incubated with primary
antibodies at room temperature for 2 h or 4°C overnight, followed by incubation with secondary antibodies at room temperature for 3 h. The results were acquired by exposure to x-ray films after treatment with a mixture of equal volumes of Luminol/Enhancer Solution and Stable Peroxide Solution (Thermo Scientific, Waltham, MA, USA). β-Actin was used as a control. The primary antibodies used were as follows: anti-HSD3B1 and anti-HSD11B1 (Abcam, Cambridge, UK, 1:1000), anti-CYP17A1 (Santa Cruz Biotechnology, Dallas, TX, USA, 1:1000) and anti-β-actin (Abcam, 1:1000). The goat anti-rabbit secondary antibodies (Abcam) were used at a dilution of 1:5000.

Statistical analyses
All experiments were conducted at least three times. The data were analyzed with SPSS Statistics 17.0 software package (SPSS, Chicago, IL, USA). P-values less than 0.05 were considered statistically significant.

Results
Dose optimization of EDS for treating male neonatal rats
To validate whether FLCs have an effect on the development and function of ALCs, we compared the development of ALCs in rat models with and without FLCs. Administration of EDS to adult and neonatal rats resulted in the destruction of all Leydig cells, followed by complete regeneration of these cells [24, 28]. However, the toxicity of different doses of EDS in neonatal rats has not been clarified [28–30]. Thus, we treated newborn rats with different doses of EDS and DMSO by single intraperitoneal injection (50 μl/rat). Following treatment with 75, 100, and 125 mg/kg EDS and with DMSO solution as a control, at 4 days after injection the rats’ survival rate, body weight, testis weight, serum testosterone, and FLC number were measured. The survival rate was decreased in the group treated with 125 mg/kg EDS, while treatment did not significantly influence the other groups (data not shown). The body/testis weight ratio was significantly decreased in the 100 and 125 mg/kg treated groups (P < 0.05) (Fig. 1c). Compared to the control group, the serum testosterone levels of EDS-treated groups were decreased (P < 0.01) (Fig. 1d). FLCs (3β-HSD-positive cells, dark blue, white arrow) in the testes were eliminated in the 100 and 125 mg/kg treated groups (Fig. 1e).

The results of survival rate, body and testis weight loss, FLC ablation effect, and serum testosterone levels showed that treatment with 100 mg/kg EDS was the best treatment dose for eliminating

![Fig. 1.](image-url) Influences of different doses of EDS (ethane dimethane sulfonate) treatment on male neonatal rats 4 days after treatment. (a) EDS affected the body weights of neonatal rats. The body weights of the 125 mg/kg EDS treated group were significantly decreased compared to that of the control group. (b) EDS affected the testis weights of neonatal rats. The testis weights of 100 or 125 mg/kg EDS-treated groups were significantly decreased compared to the weights of the control group. (c) Testis weight/body weight ratio (TW/BW ratio). TW/BW ratios of 100 or 125 mg/kg EDS-treated group were significantly decreased compared to in the control group. (d) EDS affects serum testosterone level in neonatal rats. Serum testosterone levels measured by radioimmunoassay were decreased in all EDS-treated groups compared to in the control group. Values are the mean ± SEM, n = 10, * P < 0.05, ** P < 0.01 compared to the control group (CON). (e) 3β-HSD immunohistochemical staining showed that FLCs (dark blue, white arrow) were ablated in the experimental groups treated with 100 or 125 mg/kg EDS.

| Table 1. QPCR primers |
|-----------------------|
| Gene     | ID (UCSC)   | Forward primer (5′–3′)       | Reverse primer (5′–3′)     |
|-----------|-------------|------------------------------|-----------------------------|
| Rps16     | NM_001169146.1 | AAGTCTTCTTCGGACGCAAGAAA     | TTGCCCAAGACGACAGAGAG      |
| Scarb1    | NM_031541.1  | ATGGTACCTGCGGGAGAGAT        | CGAACACCTTGATCTGGTGTA      |
| Star      | NM_031558.2  | CCCAAATGCAAGAAAATCCA        | AGGCACTCCTCCAAAAGTG        |
| Nr5a1     | NM_001191099.1 | CAGAGTCGCAAATCGACAAA        | CCGGAACTCGTCTGGTTTCTC      |
| Hsd3b1    | NM_001007719.3 | CCGTGCCTACTGGCTTGC         | TCTGGCTTCGTTCTCCC          |
| Hsd17b3   | NM_054007.1  | TTCCCTCGGAGAGAGGG      | TCGCAGCGGGCTCGTGGTCG      |
| Cyp17a1   | NM_012753.1  | TGGCTTTCTCCTGCTGCCAACATC   | TCAAGGTTGAGTCTGCCTGGAAG    |
| Lhcg      | NM_012978.1  | TAAACACAGCGATCCGGACCCC    | GTGAAGGAAAGACAGGGCGC      |
| Hsd11b1   | NM_017080.2  | TCGGTAGGAGATGCTCAAGGA      | AGGCGACACTAGCCAACCTTC      |
FLCs with the least harm to neonatal rats. Therefore, 100 mg/kg EDS treatment was used in further analyses.

Effect of EDS on male neonatal rats at different times

After treatment with 100 mg/kg EDS, weights of the rat body, testis, and seminal vesicle were measured at different time points (Fig. 2). All rats survived. No significant differences were observed between the experimental and control groups in body weight at different times (Fig. 2a), while a temporary decrease in testis weight after EDS treatment was observed (Fig. 2b). SV weight was also affected by EDS. EDS-treated rats suffered from SV weight loss at 56 days post-EDS injection (Fig. 2c), while no significant differences were detected at the other time points. Although there was a temporary impact on the rat testis, testis weight increased over days 21–35. The results showed that the toxicity of EDS treatment was low on the rats’ short-term health.

Regeneration of Leydig cells in neonatal rat testis after EDS treatment

It was clear that SLCs existed in postnatal rat testis and could lead to Leydig cells regeneration through a differentiation pathway of SLC → PLC → ILC → ALC [19, 24]. To determine the ablation effects and regenerated cell number of FLCs, cell morphology, and cell type, immunohistochemistry and immunofluorescence were conducted. Leydig cells were stained with the markers 3β-HSD, 17β-HSD, and 11β-HSD1. 3β-HSD is a biomarker for all Leydig cells, while 17β-HSD and 11β-HSD1 are markers of ALC [31, 32]. Additionally, ALCs can be observed on day 56 postpartum [17, 20], which is consistent with the results observed in the control group. Therefore, HSD11B1-positive cells are likely to be mature ALCs (Fig. 3c).

Function of newly formed Leydig cells

The main function of Leydig cells is to produce steroid hormones, particularly testosterone [34]. The function of the newly formed cells was evaluated by ALC population size and testosterone concentration measurement (Fig. 4). Our results showed that compared to the control group, the serum testosterone levels first increased and then decreased (Fig. 4a). On day 56 post-EDS treatment, the serum testosterone level in the experiment group was significantly lower than in the control group. On day 56 after treatment, the samples were stained with HSD11B1 (a marker of ALCs) (Fig. 4b). Our results showed that the EDS group had a similar percentage of HSD11B1-positive cells as the control group, indicating that the EDS group had a similar ALC population size as the control (Fig. 4d). Although the ALC population size showed no significant difference, the testosterone/cell number ratio was significantly lower than in the control group (Fig. 4e). These data indicate that the function of ALCs which appeared after neonatal EDS treatment was attenuated and had a weaker capacity for testosterone production.

Mechanism leading to lower testosterone levels after EDS treatment in neonatal rats

The beta subunit of luteinizing hormone (LH), which is encoded by Lhb, is expressed in the pituitary gland and contributes to hormonal regulation [35]. As previously described, EDS treatment eliminated all FLCs in neonatal rat testis and led to decreased serum testosterone levels and increased serum LH levels [36]. To understand how the expression of Lhb responds to a low level of serum testosterone, we measured the Lhb mRNA level in the rat models. Because of the difficulty in isolating the small pituitary glands in the newborn rats, the whole brain was used for Lhb detection and total RNAs extracted from the rat brain samples were used to detect Lhb mRNA levels. Our results revealed no significant difference in Lhb RNA levels
Fig. 3. Regeneration of Leydig cells in the testis of male rats after EDS treatment. (a) 3β-HSD immunohistochemical staining in rat testes. Both FLCs and PLCs were stained with the cytoplasmic steroidogenic enzyme marker 3β-HSD (positive: dark blue; FLCs (narrow white arrow) are large and round, PLCs (wide white arrow) are spindle-shaped). 3β-HSD staining showed that FLCs were eliminated on day 4 after EDS treatment, while PLCs were generated 7 days after EDS treatment. (b) 17β-HSD immunohistochemical staining in rat testes. 17β-HSD staining showed that FLCs were eliminated after EDS treatment, and PLCs were generated earlier than in the control group after EDS treatment. (c) HSD11B1 immunofluorescence staining in rat testes. HSD11B1 immunofluorescence staining showed an obvious decrease in ALCs after EDS treatment compared to in the control groups. CON: Control group treated with DMSO solution, EDS: Treated with 100 mg/kg EDS.
Most steroidogenic enzyme genes are involved in regulating testosterone synthesis [37]. Thus, we predicted that FLC ablation would lead to altered or inactivated expression of some steroidogenic enzymes, and thus contribute to decreasing testosterone levels. The expression of the main steroidogenic enzymes was measured to test this prediction. After single injection of 100 mg/kg EDS or DMSO, the mRNA levels of steroidogenic enzyme genes *Scarb1* (scavenger receptor class B), *Star* (steroidogenic acute regulatory), *Hsd3b1*, *Cyp17a1*, *Hsd17b3*, and *Hsd11b1* [37] and male secondary sexual character development-related genes luteinizing hormone/choriogonadotropin receptor (*Lhcgr*) and nuclear receptor subfamily 5 group a member 1 (*Nr5a1*) [38, 39] were measured at 4, 7, 14, 21, 35, and 56 days. The mRNA levels of *Lhcgr*, *Star*, *Hsd3b1*, *Cyp17a1*, *Hsd17b3*, *Scarb1*, and *Nr5a1* were higher than those in the control group for the first 21 days after EDS treatment, but gradually decreased thereafter. After 56 days of treatment, there were no significant differences (P > 0.05) between the experimental and control groups in the mRNA levels of *Lhcgr*, *Star*, *Hsd3b1*, *Cyp17a1*, *Hsd17b3*, *Scarb1*, and *Nr5a1* (Fig. 5a). The protein levels of HSD11B1 and steroidogenic enzymes HSD3B1 and CYP17A1 were also measured. Compared to the control group, HSD11B1 was dramatically decreased at 56 days after EDS treatment. Consistent with the changes in the above mRNA levels, the protein levels of HSD3B1 and CYP17A1 were decreased for the first 21 days, but then increased after 35 days (Fig. 5b and c).

**Discussion**

Leydig cells in the interstitial tissue of the testis play pivotal roles in the synthesis of steroid hormones, including testosterone [23]. Studies have shown that there are at least two types of Leydig cells, FLCs and ALCs [40]. Previously, FLCs and ALCs were thought to be present in the fetal and adult testis, respectively [40]. However, a recent study suggested that FLCs persist in the testis until the adult stage and differentiate into HSD17B3- and HSD3B6-negative (steroidogenic enzymes) cells in adult testis [12, 30]. Leydig cell differentiation is strictly regulated by numerous factors, such as basic fibroblast growth factor [41], LH [42, 43], steroidogenic factor 1 [43], and Sertoli cells [44]. Although ALCs clearly do not arise from FLCs, the effect of FLCs on the development of ALCs is poorly understood.

EDS exhibits specific Leydig cell cytotoxicity and is widely used in the testis to study Leydig cell development (reviewed in detail in [45]). Researchers have used EDS to completely eliminate the LC population in either neonatal or adult rat testes and then study the regeneration of LCs after EDS treatment [20, 45, 46]. Although FLCs in neonatal rats or ALCs in adult rats are dispelled in by 72 h post-EDS treatment, previous studies suggested that EDS treatment does not affect either the origin of ALCs or subsequent development of ALCs between the experimental and control groups (Fig. 4c).

Fig. 4. Functional change of ALCs after EDS treatment. (a) Serum testosterone concentration measured by radioimmunooassay in male neonatal rats after EDS treatment. Compared to the control group, the serum testosterone concentration was significantly decreased in the first 7 days, and then significantly increased on day 14 post-EDS treatment, followed by a dramatic decrease at 56 days post-EDS treatment. Values are the means ± SEM, n = 6. * P < 0.05,** P < 0.01 compared to the control group. (b) ALCs stained with HSD11B1 (ALCs were dark blue). (c) mRNA levels of Lhb showed no significant difference between the experimental and control groups in RT-PCR. (d) Percentage of ALCs in 56-day-old rat testis. There was no significant difference between the control and EDS-treated group. (e) Testosterone/ALC number ratio showed that testosterone production was attenuated in the EDS group. Values are the means ± SEM, n = 3. * P < 0.05, compared to control group.
RAT FLCs INFLUENCE ALC DEVELOPMENT

Additionally, at approximately 14–21 days post-EDS treatment, the seminiferous epithelium was grossly abnormal, while by 48 days post-EDS treatment, spermatogenesis returned to normal [48]. Thus, EDS treatment is a good research model of Leydig cell development with no effects on spermatogenesis. Studies of the EDS elimination effect on ALCs showed that EDS induced apoptosis of ALCs by activating Fas [49]. Later studies suggested that EDS exerted its cytotoxic effects by impairing the steroidogenic capacity of the mitochondria [50]. However, whether the mechanism of EDS elimination on FLCs is the same is unknown.

We confirmed that EDS has a FLC-specific ablation effect in the newborn rat testis. Seven days after treatment, some 3β-HSD-positive (Leydig cell-specific marker) cells were observed. Because of the spindle shape character and HSD3B1-positive staining as shown in Figure 3, the newly formed Leydig cells were likely PLCs. To confirm this speculation, the mRNA levels of Lhcgr, Hsd3b1, and Hsd11b1 were measured (Fig. 5b and c). Lhcgr and Hsd3b1 were significantly upregulated in the EDS-treated group compared to in controls, while Hsd11b1 exhibited an acute decrease at 7–21 days after EDS treatment. These results are consistent with the characteristics of PLCs (Lhcgr- and Hsd3b1-positive, Hsd17b3-weakly positive, and Hsd11b1-negative). Furthermore, the population of PLCs appeared to increase compared to in the control group on days...
21 or 35 post-EDS treatment. These data suggest that eliminating FLCs in neonatal rat testis leads to accelerated development of PLCs. Little is known about the regulation and mechanism of regenerated SLC differentiation into PLCs and PLCs differentiation into ILCs. Previous research suggested that LH can stimulate the differentiation of SLCs in the presence of macrophages [51, 52]; however, this process can also occur in the absence of LH [52, 53]. Therefore, locally produced factor may affect the differentiation of PLCs to PLGCs [45]. Further studies are needed to determine the detailed mechanism of the acceleration of PLC development after FLC elimination by EDS.

The production of testosterone is affected by EDS treatment. Our data also showed that serum testosterone levels increased and reached a peak on the 14th day post-EDS treatment, and then decreased, as shown in Fig 4a. The increasing level of serum testosterone may originate from the secretion of abundant newly formed PLCs. Androstenedione produced by PLCs may replace that secreted by FLCs and become transformed into testosterone by Sertoli cells. However, the decreased levels of testosterone after EDS indicate abnormal development of ALCs in the treatment group. The mRNA levels of Lhb and steroidogenic-enzyme genes showed no significant differences between experimental and control groups at 56 days after EDS treatment (Fig. 4 and Fig. 5), which is consistent with the former results. Thus, testosterone synthesis was reduced by the limited effect of Lhb and steroidogenic enzymes in this study. Combining the results of q-PCR and western blotting with serum testosterone levels, our results indicate that the function of ALCs was attenuated by neonatal EDS treatment.

Previous studies suggested that circulating testosterone levels influence Leydig cell progenitor development through Notch signaling [54]. However, our data revealed no differences in the cell population of ALCs at 56 days after EDS treatment (Fig. 4d). However, immunofluorescence staining (Fig. 3c), Q-PCR, and western blotting (Fig. 5) showed that at 56 days after EDS treatment, the expression level of HSD11B1, an ALC marker and redox generator in testosterone biosynthesis in Leydig cells [33], was significantly decreased compared to the control group. Collectively, our study showed that the abnormal development of ALCs led to decreased testosterone levels. It remains unknown whether FLC ablation or other causes led to ALC dysfunction. Evidences indicated that other factors such as LH and steroidogenic factor 1 may also be attributed to the acceleration of PLC development after FLC elimination by EDS.

Acknowledgment

This study was supported by the Support Program of Science and Technology Department of Sichuan Province (Grant No.: 2014SZ0031).
RAT FLCs INFLUENCE ALC DEVELOPMENT

231

adul testis. FASEB J 2015; 29: 2327–2337. [Medline] [CrossRef]

Killoone KB, Smith LB, Atanassova N, Macpherson N, McKinnell C, van den Drissche S, Jobling MS, Chambers TJ, De Gendt K, Verhoeven G, Olhava I, Platts S, Renato de Franca L, Lara NL, Anderson RA, Sharpe RM. Fetal programming of adult Leydig cell function by androgenic effects on stem/progenitor cells. Proc Natl Acad Sci USA 2014; 111: E1924–E1932. [Medline] [CrossRef]

Heng K, Anand-Ivell R, Teerds K, Ivell R. The androgenic disruptors dibutyl pthalate (DBP) and diethylstilbestrol (DES) influence Leydig cell regeneration following ethane dimethanesulphonate treatment of adult male rats. Int J Androl 2012; 35: 353–363. [Medline] [CrossRef]

Kerr JB, Donachie K, Rommerts FF. Selective destruction and regeneration of rat Leydig cells in vivo. A new method for the study of seminiferous tubular-interstitial tissue interaction. Cell Tissue Res 1985; 242: 145–156. [Medline] [CrossRef]

Jackson AE, OLeary PC, Ayers MM, de Kretser DM. The effects of ethylene dimethane sulphonate (EDS) on rat Leydig cells: evidence to support a connective tissue origin of Leydig cells. Biol Reprod 1986; 35: 425–437. [Medline] [CrossRef]

Rishbridger G, Kerr J, de Kretser DM. Differential effects of the destruction of Leydig cells by administration of ethane dimethane sulphonate to postnatal rats. Biol Reprod 1989; 40: 801–809. [Medline] [CrossRef]

Zaidi A, Lendon RG, Dixon JS, Morris ID. Abnormal development of the testis after administration of the Leydig cell cytotoxic ethylene-1,2-dimethanesulphonate to the immature rat. J Reprod Fertil 1988; 82: 381–392. [Medline] [CrossRef]

Kerr JB, Knell CM. The fate of fetal Leydig cells during the development of the fetal and postnatal rat testis. Development 1988; 103: 335–544. [Medline]

Ge RS, Hardy MP. Variation in the end products of androgen biosynthesis and metabolism during postnatal differentiation of Leydig cells. Endocrinology 1998; 139: 3787–3795. [Medline] [CrossRef]

Stojkov NJ, Janic MM, Bjeljic MM, Mihajlovic AI, Kostic MM, Andric AI, Dorić MM, Kostic TS, Grozdanov AJ, Mitrevski AI, Radevski JK, Kostic MM, Rinka DM, Daxisheva JD. Molecular regulation of steroidogenesis in endocrine Leydig cells. Steroids 2015; 103: 3–10. [Medline] [CrossRef]

Tremblay JJ. Molecular regulation of steroidogenesis in endocrine Leydig cells. Steroids 2015; 103: 3–10. [Medline] [CrossRef]

Juergenss JS, Quirk CC, Nilson JH. Multiple and overlapping combinatorial codes orchestrate hormonal responsiveness and dictate cell-specific expression of the genes encoding luteinizing hormone. Endocr Rev 2004; 25: 521–542. [Medline] [CrossRef]

Thomson SD, Lendon RG, Morris ID. Testicular effects of the Leydig cell toxicant ethane dimethanesulphonate given to neonatal rats. Reprod Toxicol 1991; 5: 497–504. [Medline] [CrossRef]

Ye L, Su ZJ, Ge RS. Inhibitors of testosteron biotransform and metabolic activation enzymes. Molecules 2011; 16: 9983–10001. [Medline] [CrossRef]

Vezzoli V, Dumincuio P, Vottero A, Kleinau G, Schleich R, Minari R, Bassi I, Bernasconi S, Persani L, Bosomi M. A new variant in signal peptide of the human luteinizing hormone receptor (LHCR) affects receptor biogenesis causing Leydig cell hypoplasia. Hum Mol Genet 2015; 24: 6003–6012. [Medline] [CrossRef]

Suntharalingham JP, Buonocore F, Duncan AJ, Achermann JC. DAX-1 (NR0B1) and steroidogenic factor-1 (SF-I, NR5A1) in human disease. Best Pract Res Clin Endocrinol Metab 2015; 29: 687–699. [Medline] [CrossRef]

Roosens-Runge EC, Anderson D. The development of the interstitial cells in the testis of the albino rat. Acta Anat (Basel) 1959; 37: 129–137. [Medline] [CrossRef]

Liu H, Yang Y, Zhang L, Liang H, Ge RS, Zhang Y, Zhang Q, Xiang Q, Huang Y, Su Z. Basic fibroblast growth factor promotes stem Leydig cell development and inhibits LH-stimulated androgen production by regulating microRNA expression. J Steroid Biochem Mol Biol 2014; 144(Pt B): 483–491. [Medline] [CrossRef]

Gao JJ, Ma X, Wang CQ, Ge YF, Lian QQ, Hardy DO, Zhang YF, Dong Q, Xu YF, Ge RS. Effects of luteinizing hormone and androgen on the development of rat progenitor Leydig cells in vitro and in vivo. Asian J Androl 2013; 15: 685–691. [Medline] [CrossRef]

Karpova T, Ravichandiran K, Insisennoy M, Rice D, Aghor V, Heckert LL. Steroidogenic factor 1 differentially regulates fetal and adult Leydig cell development in male mice. Biol Reprod 2015; 93: 83. [Medline] [CrossRef]

Reboursect D, OShaughnessy PJ, Pitetti JL, Monteiro A, Olhava L, Milne L, Tasi YT, Cruickshanks L, Rethemacher D, Guillou F, Mitchell K, van't Hof R, Freeman TC, Ne S, Smith LB. Sertoli cells control peritubular myoid cell fate and support adult Leydig cell development in the prepubertal testis. Development 2014; 141: 2139–2149. [Medline] [CrossRef]

Teerds K, Rijntjes E. Dynamics of Leydig cell regeneration after EDS. In: Payne AH, Hardy MP (eds.), The Leydig Cell in Health and Disease. Totowa, NJ: Humana Press; 2007: 91–116.

Teerds KJ, de Boer-Brouwer M, Dorrington JH, Ravters M, Ivell R. Identification of markers for precursor and Leydig cell differentiation in the adult rat testis following ethane dimethyl sulphonate administration. Biol Reprod 1999; 60: 1437–1445. [Medline] [CrossRef]

Edwards G, Jackson H, Morris ID. Testicular endocrine effects of alkyl methane-sulfonates related to the Leydig cell cytotxic compound. EDS. Cancer Chemother Pharmacol 1990; 26: 19–25. [Medline] [CrossRef]

Morris ID, Phillips DM, Bardwin CW. Ethylene dimethanesulfonate destroys Leydig cells in the rat testis. Endocrinology 1986; 118: 709–719. [Medline] [CrossRef]

Taylor MF, de Boer-Brouwer M, Woolveridge I, Teerds KJ, Morris ID. Leydig cell apoptosis after the administration of ethane dimethanesulfonate to the adult male rat is a Fas-mediated process. Endocrinology 1999; 140: 3797–3804. [Medline] [CrossRef]

King SR, Rommerts FF, Ford SL, Hutson JC, Orly J, Stoco DM. Ethane dimethane sulfonate and NNNN-tetrakis-(2-pyridylmethyl)ethylenediamine inhibit steroidogenic acute regulatory (STAR) protein expression in MA-10 Leydig cells and rat Sertoli cells. Endocr Res 1998; 24: 469–478. [Medline] [CrossRef]

Molenaar R, de Rooy DJ, Rommerts FF, van der Molen HJ. Repopulation of Leydig cells in mature rats after selective destruction of the existing Leydig cells with ethylene dimethane sulfonate is dependent on luteinizing hormone and not follicle-stimulating hormone. Endocrinology 1986; 118: 2546–2554. [Medline] [CrossRef]

Teerds KJ, de Rooy DJG, Rommerts FF, Wensing CJ. The regulation of the proliferation and differentiation of rat Leydig cell precursor cells after EDS administration or daily HCG treatment. J Androl 1988; 9: 343–351. [Medline] [CrossRef]

Teerds KJ, de Rooy DJG, Rommerts FF, van den Hurk R, Wensing CJ. Proliferation and differentiation of possible Leydig cell precursors after destruction of the existing Leydig cells with ethane dimethyl sulfonate: the role of LH/human chorionic gonadotrophin. J Endocrinol 1989; 122: 689–696. [Medline] [CrossRef]

Daf talo T, Sarawathula A, Brot A, Iruela-Arispe ML, Capel B. Testosterone levels influence mouse fetal Leydig cell progenitors through notch signaling. Biol Reprod 2013; 88: 91. [Medline] [CrossRef]