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—Original Article—

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

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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

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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

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and HSD11B1-negative [19–21]. Thus, PLCs can also secrete steroids, mainly androsterone. ILCs are round, rich in lipids [21], and secrete high levels of 5α-reduced androgens. In contrast to PLCs or ILCs, ALCs are the largest cells among this cell lineage, contain abundant smooth endoplasmic reticulum, and secrete high levels of testosterone [21]. They are HSD3B1-, HSD17B3-, HSD11B1-, and CYP17A1-positive [20]. The relationship between FLCs and ALCs remains unclear. Some researchers have suggested that FLCs and ALCs share common precursors, while others suggested that the two cell types arise from different precursors [15, 18]. Moreover, little is known about the influence of FLCs on the development of ALCs.

The main circular testosterone in adult males is secreted by ALCs. Moreover, 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 to prevent the needle from damaging the abdominal viscera. 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 ddH_2O, 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-μ 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

Table 1. QPCR primers

Gene	ID (UCSC)	Forward primer (5'–3')	Reverse primer (5'–3')
Rps16	NM_001169146.1	AAGTCTTCTTCGGACGCAAGAAA	TTGCCCAGAAGCAGAACAG
Scarb1	NM_031541.1	ATGGTACTGCCGGGCAGAT	CGAACACCCTTGATTCCTGGTA
Star	NM_031558.2	CCCAAATGTCAAGGAAATCA	AGGCATCTCCCCAAAGTG
Nr5a1	NM_001191099.1	CAGAGCTGCAAAATCGACAA	CCCGAATCTGTGCTTTCTTC
Hsd3b1	NM_001007719.3	CCCTGCTCTACTGGCTTGC	TCTGCTTGGCTTCCTCCC
Hsd17b3	NM_054007.1	TTTCTTCGGGAGTAGGGGTTC	TCATCGGCGGTCTTGGTCG
Cyp17a1	NM_012753.1	TGGCTTTCCTGGTGCACAATC	TGAAAGTTGGTGTTCGGCTGAAG
Lhcgr	NM_012978.1	TAACACAGGCATCCGAACCC	GTGAGTAGGAAGACAGGGCG
Hsd11b1	NM_017080.2	TCGGTAGGAGATGCTCAGGA	AGGCAGCACTAGCCAACTTC

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 affect 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 testis 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

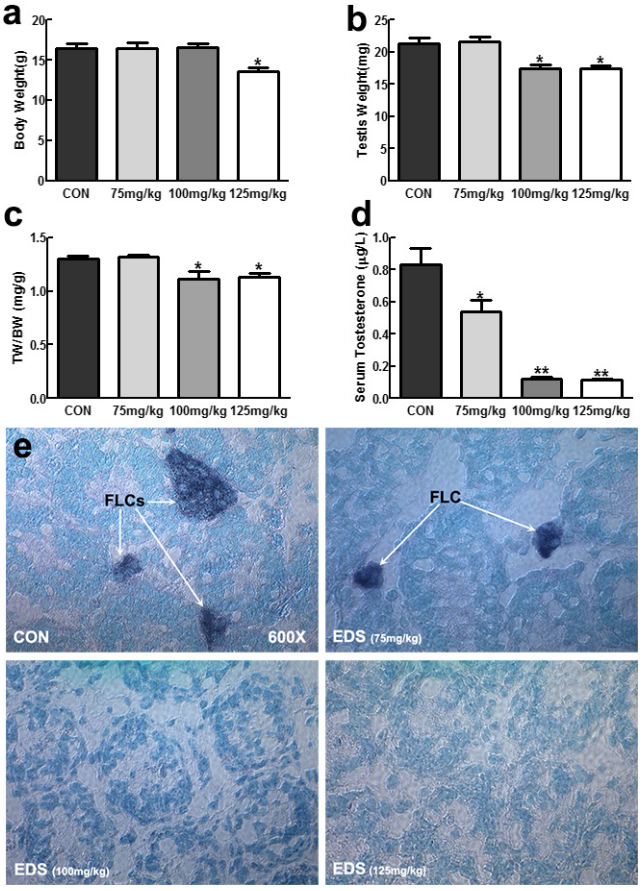

Fig. 1. Influences of different doses of EDS (ethane dimethane sulfonate) treatment on male neonatal rats 4 days after treatment. (a) EDS affected the body weights of neonatal rats. The body weights of the 125 mg/kg EDS treated group were significantly decreased compared to that of the control group. (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.

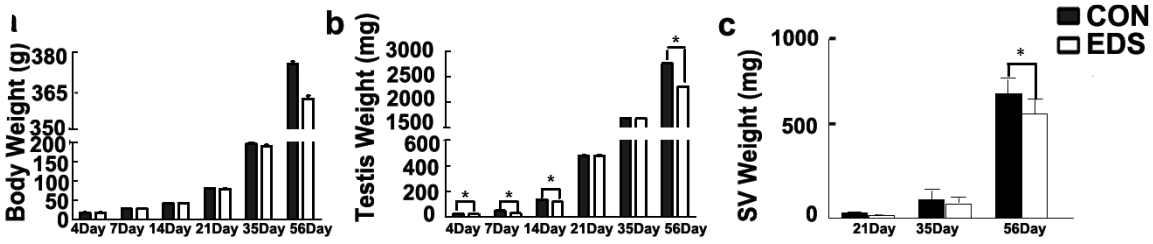

Fig. 2. Relative body, testis, and seminal vesicle (SV) weights of male neonatal rats after EDS treatment (100 mg/kg) at different time points. (a) Body weights at different times. The body weights showed no significant differences at all time points. (b) Testis weights showed a temporary decrease, followed by an increase, while a significant decrease was observed at 56 days after EDS treatment compared to in the control groups. (c) Seminal vesicle (SV) weights were significantly decreased at 56 days after EDS treatment compared to in the control groups. Values are the mean ± SEM, n = 6, * $P < 0.05$ compared to the control group. CON: Control group treated with DMSO solution; EDS: 100 mg/kg treatment group.

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].

Our results revealed no 3β-HSD-positive cells (Fig. 3a) after 4 days of EDS treatment, suggesting that all FLCs were eliminated. Interestingly, on day 7 post-EDS treatment, some spindle-shaped cells were detected in 3β-HSD-stained samples in the experimental group, while in the control group, spindle-shaped cells were not observed, but round, large, dark blue, 3β-HSD-positive FLCs were observed until day 14 (Fig. 3a). 17β-HSD$^+$, spindle-shaped cells were observed on day 14 in the EDS group, while the same morphology and 17β HSD$^+$ cells were observed until day 21 in the control group. However, on days 7 and 14, some 17β-HSD$^+$ cells showed similar morphology as FLCs (round, large, dark blue). Our results showed that after eliminating FLCs by EDS treatment, Leydig cells were regenerated, and these newly formed cells were likely PLCs, the precursor of ALCs, based on their morphology, 3β-HSD character, and observed time [11, 20]. HSD11B1 immunofluorescence staining showed a remarkable decrease in HSD11B1 expression on day 56 post-EDS treatment compared to in the control group. HSD11B1 is a marker of ALC and plays key roles in testosterone biosynthesis [32, 33]. 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

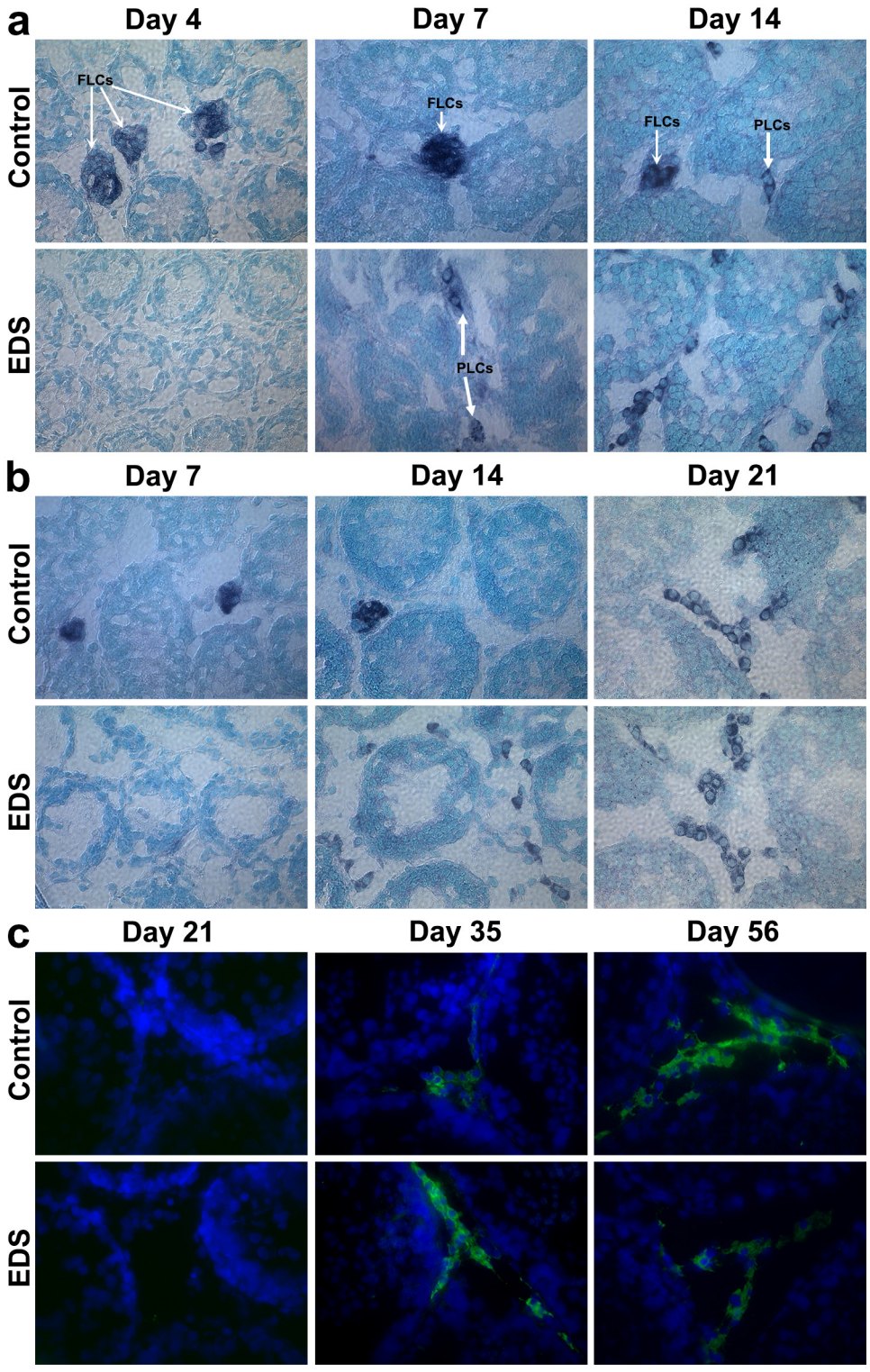

Fig. 3. Regeneration of Leydig cells in the testis of male rats after EDS treatment. (a) 3β-HSD immunohistochemical staining in rat testes. Both FLCs and PLCs were stained with the cytoplasmic steroidogenic enzyme marker 3β-HSD (positive: dark blue; FLCs (narrow white arrow) are large and round, PLCs (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.

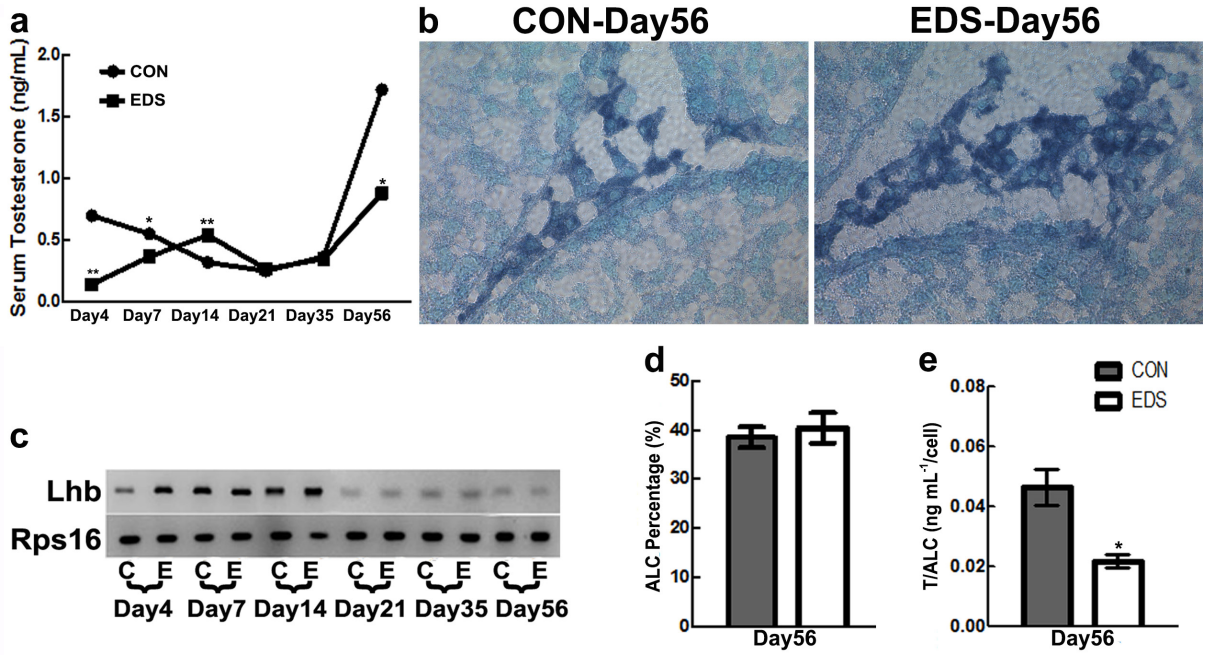

Fig. 4. Functional change of ALCs after EDS treatment. (a) Serum testosterone concentration measured by radioimmunoassay in male neonatal rats after EDS treatment. Compared to the control group, the serum testosterone concentration was significantly decreased in the first 7 days, and then significantly 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.

between the experimental and control groups (Fig. 4c).

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*, and *Scarb1* (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 ALC

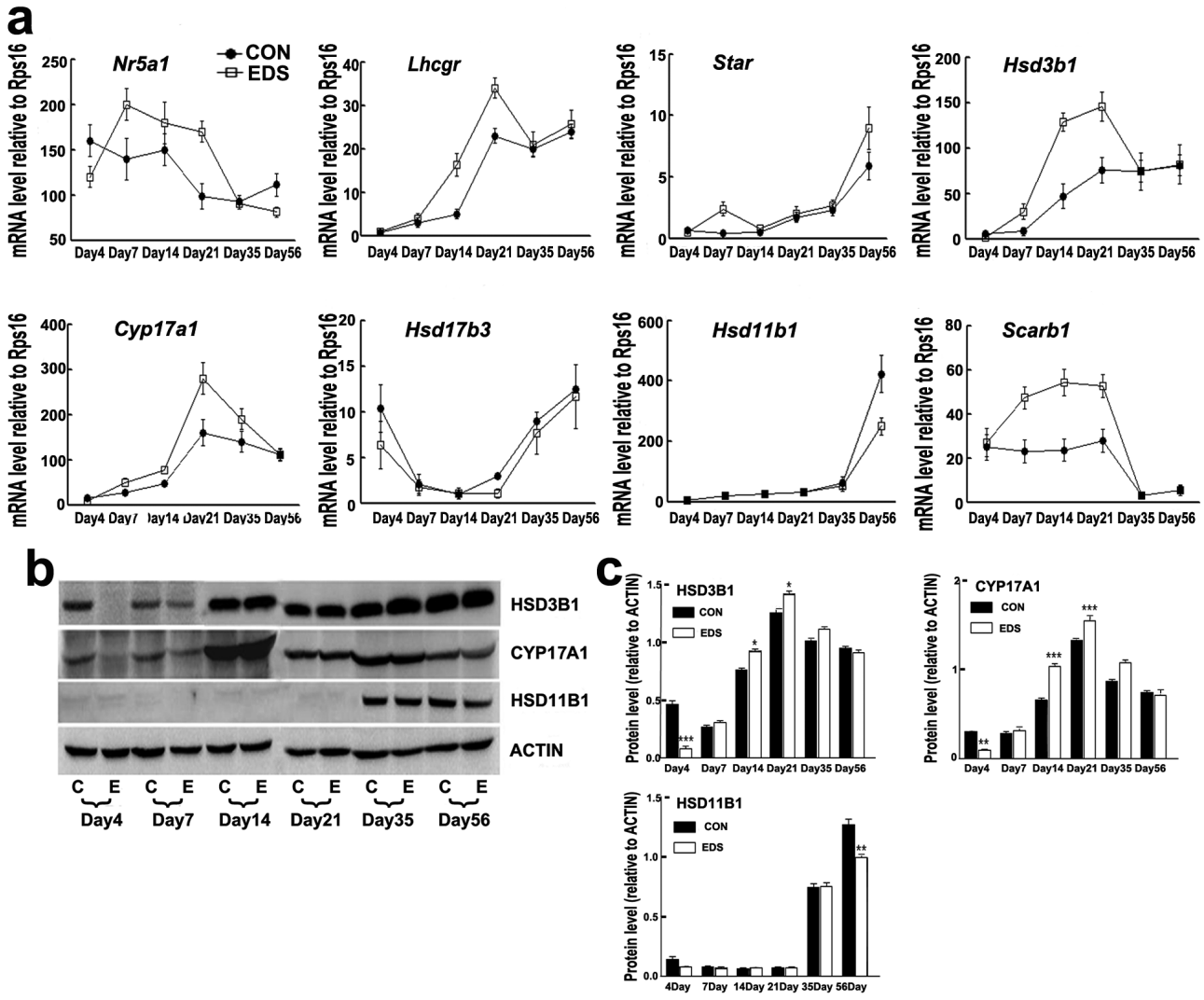

Fig. 5. mRNA and protein levels of steroidogenic-enzyme genes and Leydig cell-specific genes after EDS treatment. (a) *Nr5a1*, *Lhcgr*, *Star*, *Hsd3b1*, *Cyp17a1*, *Hsd17b3*, *Hsd11b1*, and *Scarb1* mRNA levels after EDS treatment. *Rps16* was used as a control mRNA. Compared to the control group, *Hsd11b1* was significantly decreased at 56 days after EDS treatment. (b) Representative western blot showing HSD3B1, CYP17A1, and HSD11B1 expression. (c) Protein levels of HSD3B1, CYP17A1, and HSD11B1. Compared to the expression level in the control groups, HSD3B1 and CYP17A1 were significantly increased and reached a peak at 21 days after EDS treatment, but showed no significant difference thereafter. Compared to expression in the control groups, HSD11B1 protein level was significantly downregulated at 56 days after EDS treatment. β-Actin was used as a control. Values are the means ± SEMs, n = 6, * $P < 0.05$, ** $P < 0.01$ compared to control group.

populations [20, 28, 46, 47]. 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*, *Hsd17b3*, and *Hsd11b1* were measured (Fig. 5b and c). *Lhcgr* and *Hsd3b1* were significantly upregulated in the EDS-treated group compared to in controls, while *Hsd17b3* exhibited an acute decrease at 7–21 days after EDS treatment. *Hsd11b1* remained at a low level until 56 days post-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 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 SLCs in the presence of macrophages [51, 52]; however, this process can also occur in the absence of LH [52, 53]. Therefore, a locally produced factor may affect the differentiation of SLCs to PLCs [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 afterward 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 in 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 development of Leydig cells.

Taken together, our results indicate that FLC elimination in neonatal rat testis affected the development of ALCs from PLCs. However, the function of the regenerated PLCs may be attenuated, which may further affect the development of ALCs derived from these PLCs and lead to testosterone deficiency and abnormal sexual gland development. As the proliferation and differentiation of Leydig cells are complex, the mechanism of the responses of ALC precursor cells after FLC elimination requires further analysis.

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