Research Article

Effects of Norepinephrine and Acetylcholine on the Development of Cultured Leydig Cells in Mice

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Few data have suggested how norepinephrine (NE) and acetylcholine (Ach) regulate the development of Leydig cells in mice at prepuberty, except for data indicating endocrine effects. The present study aims to elucidate the roles of NE and Ach on the differentiation and proliferation of Leydig cells. Firstly, the expression of adrenergic receptors and muscarinic acetylcholine receptors in Leydig cells was investigated. It was found that adrenergic receptors (β1AR, β2AR, and α1D) and muscarinic acetylcholine receptors (M1 and M3) mRNA are expressed in adult Leydig cells. Then, the effects of NE and Ach on the differentiation and proliferation of Leydig cells were analyzed. The results showed that NE and Ach at $10\,\mu$M significantly increased the number of 3β-hydroxysteroid-dehydrogenase- (3β-HSD-) positive Leydig cells and improved the expression of proliferating cell nuclear antigen (PCNA) in Leydig cells on postnatal day (PD) 15 ($P < 0.05$). NE and Ach at $10\,\mu$M had no impact on the expression of PCNA mRNA ($P > 0.05$), but reduced the expression of 3β-HSD mRNA in adult Leydig cells and a murine Leydig tumor cell line (MLTC-1) ($P < 0.05$). Therefore, a conclusion may be reached that NE and Ach participated in stimulating the development of Leydig cells in mice from prepuberty to adult stage.

1. Introduction

It has been generally acknowledged that Leydig cells play a key role in synthesizing testosterone and regulating the spermatogenesis. The final population of Leydig cells in the adult testis is established depending on the proliferation and differentiation of Leydig cells at prepuberty and develops to the adult population of Leydig cells [1, 2]. The adult Leydig cells develop from stem Leydig cells through several steps of differentiation and proliferation which occur during prepuberty [3]. Compared with the endocrine effects, previous data seldom demonstrate the effects of norepinephrine (NE) and acetylcholine (Ach) on the development of Leydig cells through their receptors.

α-Adrenergic and β-adrenergic receptors may be subclassified into three receptors, that is, α1A, α1B, α1D, and β1AR, β2AR, β3AR, respectively. β2AR is more widely expressed [4–6]. Muscarinic acetylcholine receptors (mAChRs) include five subtypes, that is, M1, M2, M3, M4, and M5 [7]. As of yet, the expression of adrenergic and muscarinic acetylcholine receptors in testicular Leydig cells has been unclear. It was indicated in some studies that catecholamine was capable of stimulating the secretion of testosterone through both alpha- and beta-adrenergic receptors in the immature testis of the golden hamster [8]. Moreover, carbachol, a cholinergic agonist, is capable of inhibiting the secretion of testosterone via purified rat Leydig cells [9, 10].

One objective of the present study is to elucidate the expression of adrenergic and muscarinic acetylcholine receptors in the testicular Leydig cells of mice. Another objective is to investigate the effects of NE and Ach on the proliferation and differentiation of Leydig cells at prepuberty.

2. Material and Methods

2.1. Animals. Male Kunming mice (Peking University Health Science Center) aged PD (postnatal days) 15 and 50 were used in this study. Mice were kept in cages with a 12/12 h light/dark cycle at the controlled temperature of $23 \pm 2°$C, with water and food provided ad libitum. All experimental

animals and designs used to achieve the purposes of this study were approved by the Chinese Association for Laboratory Animal Sciences.

2.2. Isolation and Purification of Primary Leydig Cells.

The testes of mice aged PD 15 and PD 50 were removed and placed in the prechilled 1 : 1 mixture of Ham's F12 medium and DMEM, then transferred to ice, with the procedure performed as previously described [16]. The testes were then decapsulated and mechanically dispersed in the medium and subjected to enzymatic digestion in the shaking water bath, using 5 mg collagenase (type IV, 213 U/mg; Worthington Biochemical Corp., Freehold, NJ, USA) in 20 mL Ham F12/DMEM (1 : 1), at 34°C for 30 min. Following this digestion procedure, the collagenase solution was diluted four times with a culture medium, then filtered through a stainless-steel mesh to separate tissue fragments. The cells were then collected via centrifugation (1,500 r/min for 10 min) and resuspended in 2 mL of the culture medium mentioned above. The Leydig cells were purified discontinuously with a four-layer Percoll density gradient (21%, 26%, 37%, and 60%). The gradient was centrifuged at 3,000 r/min for 30 min at 4°C. The interface between 37% and 60% was collected and washed with the medium to remove Percoll. The cells isolated from the PD 15 mice were progenitor cells and immature Leydig cells. The cells isolated from the PD 50 mice were adult Leydig cells, and the purity of the Leydig cells examined by histochemical staining for 3β-HSD using 1 mg/mL etiocholanolone as the enzyme substrate was observed to be 85%. Some of the purified Leydig cells were used for detecting the expression of adrenergic and muscarinic acetylcholine receptors, and others for Leydig cell culture.

2.3. Cell Culture and Treatment.

The purified Leydig cells were placed in 24-well (1 × 10^5 cells/mL/well) plates with Ham's F12/DMEM culture medium supplemented with 10% charcoal-stripped fetal bovine serum and antibiotics. Culture was performed at 34°C in a controlled humidified atmosphere with 5% CO$_2$ in the air supply. After remaining for 24 h in the culture, the cells were washed twice with a serum-free medium, and then treated with different doses of NE (0.1 μM, 1 μM, 10 μM, and 100 μM) and Ach (0.1 μM, 1 μM, 10 μM, and 100 μM) for 24 h. After the incubation, the Leydig cells were collected from the 24-well plates.

2.4. MTT Cell Viability Assay.

After the cells were treated with different doses of NE and Ach for 24 h, the culture medium was filtered out and the cells were harvested, re-suspended in 200 μL 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide (MTT) working solution (5 mg/mL methylthiazolyldiphenyl-tetrazolium bromide in PBS), then incubated for 2 h at 37°C. The converted dye was solubilized with 3 mL acidic isopropanol (0.04 M HCl in absolute isopropanol), the absorbance of which was measured at a wavelength of 570 nm with a background subtraction at 650 nm [17].

2.5. Total RNA Extraction and Semiquantitative RT-PCR.

Total RNA was extracted from the adult Leydig cells and prepuberty Leydig cells using Trizol (Invitrogen, Carlsbad, CA, USA). Two micrograms of total RNA were reversely transcribed using 200 U of Superscript II Rnase H-Reverse Transcriptase (Gibco BRL, Bethesda, MD, USA) in a 25 μL reaction volume in the presence of 10 μM Oligo (dT), first strand buffer (50 mM Tris-HCl, 75 mM KCl, and 3 mM MgCl$_2$), 0.01 M dithiothreitol, and 10 mM of each dATP, dGTP, dCTP and dTTP. The mixture of RNA and Oligo dT was heated at 65°C for 5 min, then cooled to 4°C. The other reagents were added and RT was performed at 42°C for 1 h.

PCR amplification of adrenergic receptors (β1AR, β2AR, β3AR, α1A, α1B, and α1D) and acetylcholine receptors (M1, M2, M3, M4, and M5), 3β-HSD, PCNA, and GAPDH (a housekeeping gene) was carried out using primer pairs from published sources (Table 1). The size of the PCR products was determined through comparison with a gene marker (100 bp DNA, Promega, Madison, WI, USA) run in parallel with RT-PCR products in 1.2% agarose gel containing ethidium bromide. The relative band intensity was quantified using a computer-assisted image analysis system (Visage 2000, BioImage, Ann Arbor, MI, USA). The integrated optical density (IOD) values for 3β-HSD, PCNA, and GAPDH in each band were normalized with the corresponding GAPDH expressions.

2.6. Immunohistochemistry.

Leydig cells isolated from the PD 15 mice were cultured on glass coverslips in the presence of 10 μM NE and Ach in 24-well plates. After remaining for 24 h in the culture, the medium was removed, the coverslips were rinsed with phosphate-buffered saline (PBS, 0.01 M, pH 7.4) and fixed in 4% paraformaldehyde in PBS for 20 min at room temperature, followed by penetration with 0.2% Triton X-100 in PBS for 15 min. Subsequently, the differentiation and proliferation of the Leydig cells were assessed through detecting the expression of 3β-HSD and PCNA via immunohistochemistry and immunofluorescence. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide (H$_2$O$_2$) in PBS for 30 min at room temperature. The coverslips were then rinsed in PBS and blocked with 10% normal donkey serum and goat serum, respectively, and incubated, respectively, with the two first antibodies, that is, mouse anti-3β-HSD polyclonal antibody (diluted 1 : 300 in PBS/BSA-c) and PCNA monoclonal antibody (diluted 1 : 2000 in PBS/BSA-c), overnight at 4°C. The coverslips were rinsed and incubated with secondary antibodies, biotinylated donkey anti-mouse, and goat anti-mouse IGg (diluted 1 : 150 in PBS; Zymed, San Francisco, CA, USA) for 3 h at room temperature. The coverslips used for detecting the expression of 3β-HSD were washed in PBS and incubated with streptavidin-labeled peroxidase complex (diluted 1 : 150 in PBS; Zymed) for 3 h at room temperature; next, the antibody was visualized with 0.6 mg/mL 3,3'-diaminobenzidine tetrachloride (DAB, Sigma-Aldrich) dissolved in PBS, to which 0.03% H$_2$O$_2$ was added (for brown staining); afterwards, the Leydig cells were counterstained, blued, and sealed with glycerine. The coverslips used for detecting the expression

TABLE 1: Primer sequences.

	Primer sequence	Product size (bp)	Annealing temp. (°C)	Reference
β1AR	F: CCGCTGCTACCACGACCCCAAG R: AGCCAGTTGAAGAAGAGCAAGAGGCG	441	60	[11]
β2AR	F: GGTTATCGTCCTGGCCATCGTGTTTG R: TGGTTCGTGAAGAAGTCACAGCAAGTCTC	468	61	v.s.
β3AR	F: TCTAGTTCCCAGCGGAGTTTTCATCG R: CGCGCACCTTCATAGCCATCAAACC	337	64	v.s.
α1A	F: GGACAAGTCAGACTCAGAGCAAGTGACG R: TATAGCCCAGGGCATGCTTGGAAGAC	404	61	v.s.
α1B	F: TTTCATGAGGACACCCTCAGCAGTACC R: CTGCCGCTGTCATCCAGAGAGTCC	452	61	v.s.
α1D	F: CGCCAAAGGAAATCCAGGGACAC R: CAGAGCGGAACTTATGGGACAGG	617	61	v.s.
M1	F: GTAAGGTGCCTGCCATCCAATC R: CGCAGCTCACTTTCTGCATTGT	417	58	[12]
M2	F: TGTCAGCAATGCCTCCGTTATG R: GCCTTGCCATTCTGGATCTTG	480	58	v.s.
M3	F: GGTGTGATGATTGGTCTGGCTTG R: GAAGCAGAGTTTTCCAGGGAG	497	62	v.s.
M4	F: TCAAGAGCCCTCTGATGAAGCC R: AGATTGTCCGAGTCACTTTGCG	477	62	v.s.
M5	F: GCTGACCTCCAAGGTTCCGATTC R: CCGTCAGCTTTTACCACCAAT	485	54	v.s.
3β-HSD	F: ACTGCAGGAGGTCAGAGCT R: GCCAGTAACACACAGAATACC	565	55	[13]
PCNA	F: TGGAATCCCAGAACAGG R: ACAGTGGAGTGGCTTTT	287	55	[14]
GAPDH	F: AAACCCATCACCATCTTCCAG R: AGGGGCCATCCACAGTCTTCT	361	54	[15]

F: forward; R: reverse.

of PCNA were incubated with 1 : 50 dilution of fluorescein isothiocyanate (FITC)-conjugated streptavidin (Southern Biotech, Birmingham, AL, USA) for 3 h without exposure to light. After rinsing, the coverslips were stained with 1 : 50 dilution of Hoechst 33258 without exposure to light for 40 min. After being rinsed in PBS for the final time, the slides were mounted with 75% glycerol in PBS. A Xuorescence microscope (Leica LB30T, Germany) equipped with a filter for FITC (Leica, I3-513808) was used for observation. Images were captured using a digital camera (Leica DFC320).

2.7. Statistical Analysis. Every step of the treatment in the same experiment and the specific experiment was repeated at least three times. All data were represented as means ± SE. Statistical significance was tested using the Student's t-test. Differences between groups were considered significant if $P < 0.05$.

3. Results

3.1. The Expression of Adrenergic and Muscarinic Acetylcholine Receptors mRNA in Leydig Cells of Adult Mouse Testis. RT-PCR results showed that β1AR, β2AR, and α1D receptors
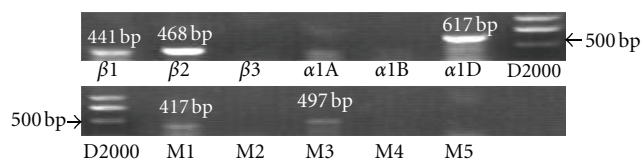

FIGURE 1: Expression of β1AR, β2AR, β3AR, α1A, α1B, and α1D receptors and M1, M2, M3, M4, and M5 receptors mRNA in Leydig cells of adult mouse testis. β1AR, β2AR, α1D, M1, and M3 receptors. PCR products of 441 bp, 468 bp, 617 bp, 417 bp, and 497 bp visualized by ethidium bromide staining are shown. D2000, size marker.

mRNA were expressed in adult Leydig cells, especially the β2AR and α1D receptor mRNA (Figure 1). β3AR and α1A receptors mRNA were not detected in the adult Leydig cells. M1 and M3 receptors mRNA were expressed in adult Leydig cells, while M2, M4, and M5 receptors mRNA were not detected (Figure 1). The data indicated that NE and Ach stimulated the development of Leydig cells through some of the receptors, that is, β1AR, β2AR, α1D, or M1 and M3.

3.2. Effects of Different Doses of NE and Ach on the Viability of Mouse Leydig Cells in PD 15 Mice. The respective viabilities
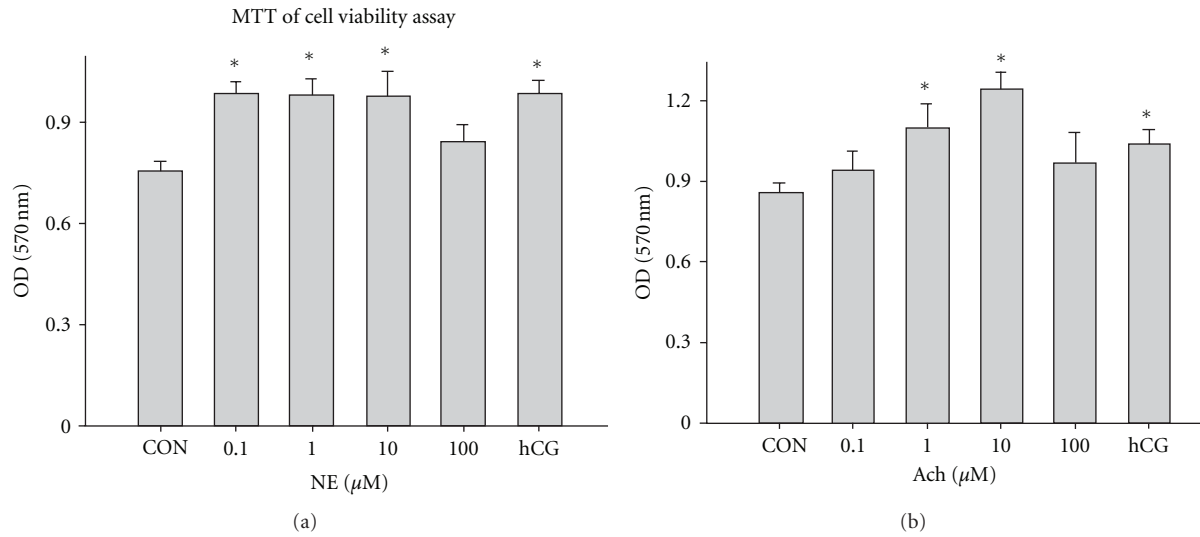
MTT of cell viability assay

(a)

(b)

FIGURE 2: NE and Ach at doses of 0.1, 1, 10, and 100 μM on the viability of Leydig cells from mice on PD15. The viability of Leydig cells incubated with different doses of NE and Ach for 24 h was assessed using MTT assays. The data were presented as means ± SEM. A single asterisks (*) indicates statistical significance ($P < 0.05$) relative to the untreated control. CON: control group cultured with the same dilution of NE and Ach. hCG: hCG group cultured with hCG at the dose of 1 IU/mL.
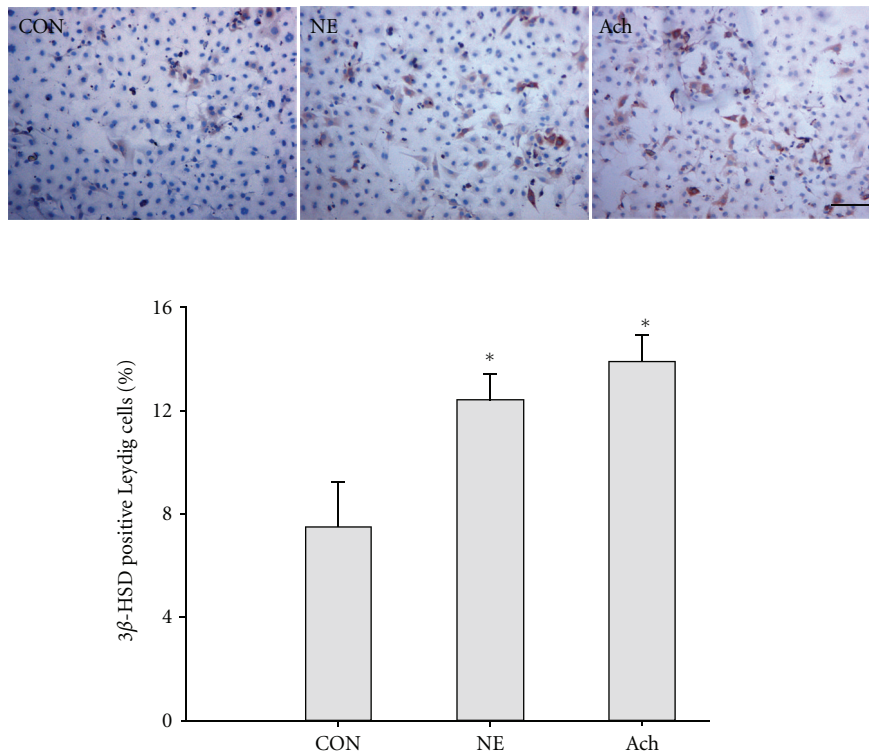

FIGURE 3: NE and Ach on the differentiation of precursor Leydig cells into 3β-HSD-positive Leydig cells. The percentage of 3β-HSD-positive cells to total Leydig cells was determined and presented as means ± SEM. A single asterisks (*) indicates statistical significance ($P < 0.05$) relative to the untreated control. CON: control group cultured with the same dilution of NE and Ach. NE: NE group cultured in the presence of 10 μM NE. Ach: Ach group cultured in the presence of 10 μM Ach. Bar = 50 μm.
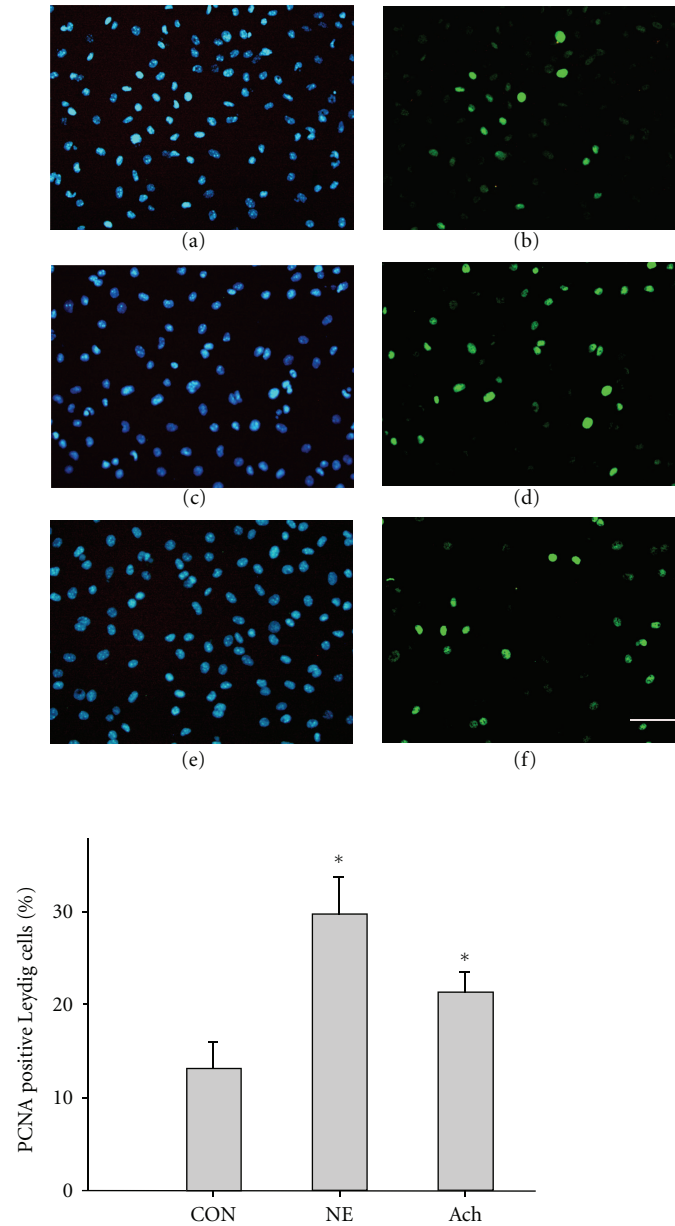
(a)

(b)

(c)

(d)

(e)

(f)

FIGURE 4: Immunofluorescence staining of PCNA in the Leydig cells cultured with NE and Ach on PD15. The percentage of PCNA-positive (green) cells to total Leydig cells (blue) was presented as means ± SEM. A single asterisks (*) indicates statistical significance ($P < 0.05$) relative to the untreated control. (a) and (b) control group cultured with the same dilution of NE and Ach. (c) and (d) NE group cultured in the presence of $10\,\mu$M NE. (e) and (f) Ach group cultured in the presence of $10\,\mu$M Ach. (b, d and f) Originated from the same photo (a), (c) and (e) with FITC staining only. Nuclei (blue) of all Leydig cells were stained with Hoechst 33258. Bar = $50\,\mu$m.

of Leydig cells in PD15 mice which were exposed to different doses of NE and Ach were observed. The results in **Figure 2** suggested that NE at $0.1\,\mu$M, $1\,\mu$M, and $10\,\mu$M significantly stimulated the growth of Leydig cells, compared with the control group ($P < 0.05$). Furthermore, Ach at $1\,\mu$M and $10\,\mu$M significantly improved the viability of Leydig cells ($P < 0.05$). Both NE and Ach with a dose of $100\,\mu$M did not stimulate the viability of Leydig cells.

3.3. Effects of NE and Ach on the Expression of 3β-HSD of Leydig Cells in PD 15 Mice. The stem Leydig cells expressed

no steroidogenic enzyme. They differentiate into progenitors, and 3β-HSD expresses between PD 10 and 13 [18]. The results showed that NE and Ach significantly stimulated the expression of 3β-HSD, compared with the control group, in the cultured Leydig cells in PD 15 mice (**Figure 3**) ($P < 0.05$). Therefore, it may be seen that NE and Ach are capable of regulating the differentiation of Leydig cells at prepuberty.

3.4. Effects of NE and Ach on the Expression of PCNA in Cultured Leydig Cells in PD 15 Mice. In order to test the effects of NE and Ach on the proliferation of Leydig cells, PCNA
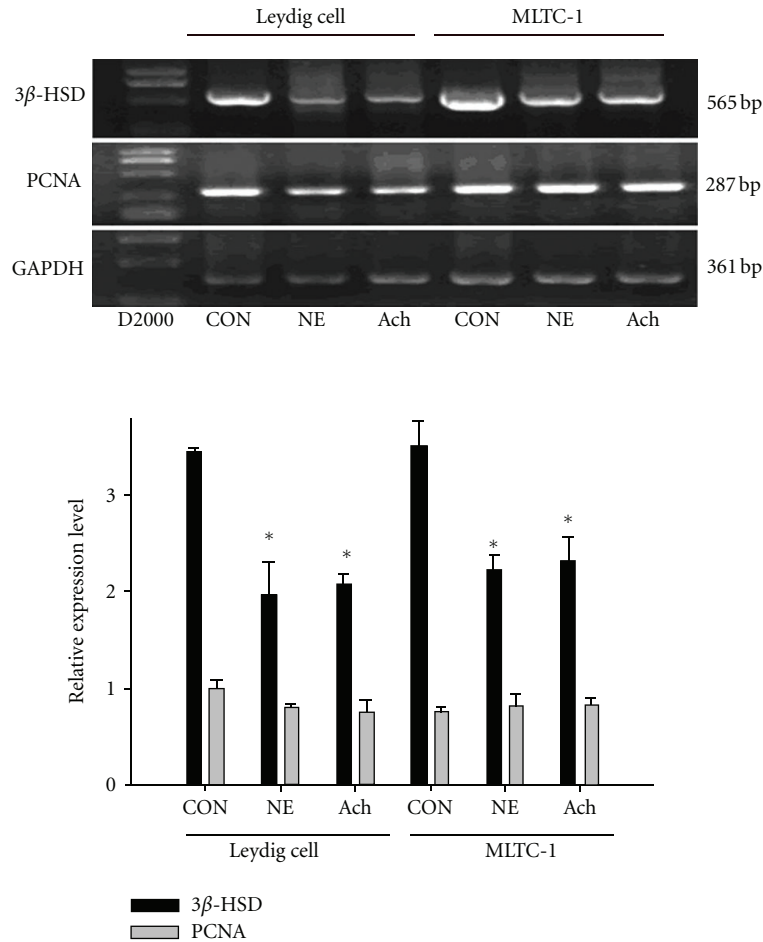
FIGURE 5: Expression levels of 3β-HSD mRNA and PCNA mRNA in the cultured adult Leydig cells and MLTC-1 treated with NE, and Ach. Asterisks (*) indicate statistical significance ($P < 0.05$) between the CON, NE and Ach groups. CON: Control group cultured with the same dilution of NE and Ach. NE: NE group cultured in the presence of 10μM NE. Ach: Ach group cultured in the presence of 10μM Ach.

immunofluorescence and cell counting were performed. PCNA-labeled cells were identified via nuclear green staining. NE and Ach significantly increased the number of PCNA-positive Leydig cells in PD 15 mice, compared with the control group ($P < 0.05$) (Figure 4). The results indicated that NE and Ach had a great impact on the proliferation of Leydig cells at prepuberty, at which time the population of adult Leydig cells was established.

3.5. Effects of NE and Ach on the Expression of 3β-HSD and PCNA mRNA in Adult Leydig Cells and MLTC-1. The RT-PCR results (Figure 5) demonstrated that NE and Ach significantly inhibited the expression of 3β-HSD mRNA in adult mouse Leydig cells and MLTC-1 ($P < 0.05$), but clear effects on the expression of PCNA mRNA were not detected ($P > 0.05$). These results indicated that NE and Ach influenced the expression of 3β-HSD mRNA, which was involved in testosterone endocrine of adult Leydig cells, but had no effect on the population of adult Leydig cells.

4. Discussion

In this study, the roles of NE and Ach involving the proliferation and differentiation of Leydig cells were investigated in vitro. The process by which NE and Ach regulated the development of Leydig cells by means of adrenergic receptors and muscarinic acetylcholine receptors was investigated. Firstly, the expression of adrenergic receptors and muscarinic acetylcholine receptors in Leydig cells was analyzed. It was found that only β1AR, β2AR, α1D, M1, and M3 receptors mRNA was expressed in adult Leydig cells, and in this way, NE and Ach regulated the function of Leydig cells. The mechanisms of the receptors modulating the development and functions of Leydig cells are a topic which still requires further study.

In order to obtain precursor cells, Leydig cells were isolated from PD 15 mice when the cell proliferation and differentiation occurred and developed to the adult population of Leydig cells [2, 3]. Different doses of NE and Ach (0.1μM–100μM) were added into the cultures in vitro. The results indicated that the viability of Leydig cells was significantly increased by NE and Ach with doses of 1 and 10μM.

Low or high exposure doses of NE or Ach did not stimulate the viability of the Leydig cells. The reason for this may be the fact that the low doses did not reach the concentration, while the high doses resulted in toxicity of the Leydig cells.

The adult Leydig cells of mice first emerge on PD 10, and the immature Leydig cells undergo proliferation and differentiation on PD 15. By the end of puberty, the development of Leydig cells is completed and the population of adult Leydig cells reaches 25×10^5 per testis [19]. In addition to LH [3], NE, and Ach also regulate the development of Leydig cells, as validated in the above results.

The 3β-HSD was used as a differentiation indicator of precursor cells [20]. Therefore, the expression of 3β-HSD was detected in the cultured Leydig cells of mouse testis treated with NE and Ach on PD 15. The results showed that NE and Ach promoted the expression of 3β-HSD in the cultured Leydig cells. Consequently, NE and Ach were shown to play an important role in the process of the stem Leydig cells differentiating into 3β-HSD- positive precursor cells.

The proliferation of Leydig cells based on PCNA expression, an indicator of proliferative activity [21, 22], was analyzed. The data showed that NE and Ach were able to facilitate the proliferation of cultured Leydig cells on PD 15. As a result, NE and Ach influenced the establishment of the population of adult Leydig cells at prepuberty. It is probable that NE and Ach stimulated the development of Leydig cells through their receptors β1AR, β2AR, α1D, M1, and M3 which were expressed in mouse Leydig cells.

In order to clarify whether NE and Ach had the same effects on adult Leydig cells, the expression of 3β-HSD and PCNA mRNA in adult leydig cells and MLTC-1 treated with NE and Ach was detected. It is worth noting that NE and Ach inhibited the expression of 3β-HSD mRNA and had no effects on the expression of PCNA mRNA in adult Leydig cells and MLTC-1. It may be presumed that the reduction of 3β-HSD mRNA was related to the testosterone endocrine of the adult Leydig cells. NE and Ach had no effect on the proliferation of the adult Leydig cells. The reason for this may be the fact that the development of adult Leydig cells had been completed at puberty [3].

5. Conclusions

It was shown that NE and Ach participated in stimulating the differentiation and proliferation of Leydig cells from prepuberty to the adult stage. NE and Ach had no effect on the population of the adult Leydig cells by the end of puberty, however, they influenced the testosterone endocrine of the Leydig cells by reducing the expression of 3β-HSD mRNA.

Acknowledgments

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