An *In Vitro* Test on Testosterone Production of Deer Leydig Cells

離體實驗研究鹿科動物萊氏細胞睪酮之生產

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Abstract

Testosterone production of Leydig cells (LC) purified from testes of the sambar deer (*Cervus unicolor swinhoei*), the sika deer (*Cervus nippon taiouanus*) and the Reeves' muntjac (*Muntiacus reevesi micrurus*) at the hard antler stage were examined *in vitro*. They were treated with various concentrations of human chorionic gonadotropin (hCG) and three steroidogenic precursors: namely, 25-OH-cholesterol, pregnenolone and androstenedione. The minimum effective concentrations for stimulating the LC testosterone productivity were determined at 0.5 IU/ml for hCG, 10^3 nM for 25-OH-cholesterol, 10^3 nM for pregnenolone, and

10^2 nM for androstenedione. All treatments resulted in detectable testosterone production after 3 days of the incubation for all three deer species tested. The results demonstrated that purified deer LC was a useful *in vitro* tool for further investigations on cervid physiology.

摘　要

本實驗純化台灣水鹿、台灣梅花鹿及台灣山羌硬角期萊氏細胞並檢測睪酮產量，將萊氏細胞處理人類絨毛膜性促素及三個前驅物，包含膽固醇、孕烯醇酮、雄脂烯二酮。在三種鹿科動物的實驗中，評估最小刺激睪酮產量的濃度依序人類絨毛膜性促素爲 0.5 IU/ml、 膽固醇爲 10^3 nM、孕烯醇酮爲 10^3 nM，以及雄脂烯二酮爲 10^2 nM。實驗結果顯示培養三天即可刺激睪酮產量，藉由本研究之成果可提供鹿科動物離體實驗初步方法學之探討。

Key words: *Cervus nippon taiouanus*, *Cervus unicolor swinhoei*, *Muntiacus reevesi micrurus*, reproductive physiology, *steroidogenesis*, *testosterone*.

關鍵詞：台灣梅花鹿、台灣水鹿、台灣山羌、生殖生理、類固醇生合成、睪酮

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Introduction

Testosterone is an important reproductive hormone produced mainly by testicular Leydig cells (LC) (Allen *et al*. 2006; Midzk *et al*. 2007). It is synthesized by a series of metabolic processes termed as "steroidogenesis" from cholesterol with stimulation of luteinizing hormone (LH) released from the anterior pituitary gland. The processes require enzymes to convert cholesterol into various forms of steroids to testosterone (Payne and Hales 2004).

Steroidogenesis starts by transferring cholesterol from the cytoplasmic pool into mitochondria. It is facilitated by the steroidogenic acute regulatory protein (StAR protein) synthesized in the cytosol. Cholesterol in the cytoplasm is transferred into the mitochondrial inner membrane. This is the most rate-limiting step for the whole steroidogenesis where the cytochrome P450 side-chain cleavage (P450scc) catalyzes the first side chain cleavage of cholesterol to yield pregnenolone. The pregnenolone is dehydrogenated to progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD), then converted to androstenedione by P450C17, and finally reduced to testosterone by 17β-oxido-reductase.

For male cervids the testosterone level in the

blood fluctuates annually in accordance with seasonal changes in the testicular activity, creating the phenomenon of "annual re-puberty," with an exception of the muntjacs (*Muntiacus* sp.) (Pei *et al*. 2009). Males become fertile and behaviorally active only during the period of high testosterone levels (Asher *et al*., 1996; Bubenik *et al*. 1996; Blottner *et al*. 1996; Goeritz *et al*. 2003; Gómez *et al*. 2006; Li *et al*. 2001; Loudon and Curlewis 1988; Monfort *et al*. 1993; Rolf and Fischer 1996; Yamauchi *et al*. 1997). The level of testosterone also dictates the antler replacement cycle. Very low level in spring initiates hard antler casting and new growth of velvet antlers. The level rises in autumn for velvet shedding and antler hardening (Gómez *et al*., 2006; Loudon and Curlewis 1988; Pei *et al*. 2009; Rolf and Fischer 1996; Scott *et al*. 1989; Yamauchi *et al*. 1997).

To have an in-depth understanding of the physiological mechanism of the "annual re-puberty" phenomenon is always a great interest to many scientists. In this study we developed and tested an *in vitro* technique for the first time to examine the mechanism of the steroidogenesis by purifying LC from testicles of three native deer species of Taiwan: the sambar deer, the sika deer, and the Reeves' muntjac. The LC was incubated *in vitro* with each of hCG and three steroidogenic precursors, namely 25-OH-cholesterol (cholesterol), pregnenolone (Δ_5P) and androstenedione (Δ_4) to examine their effects on the testosterone production.

Materials and Methods

Fresh testicles of healthy adult male sambar deer, sika deer and Reeve's muntjac with hard antlers were obtained from deer farms in the central Taiwan in 2007 and 2008. The testicular interstitial cells were isolated with the collagenase dispersion method (Tsai *et al*. 2003). They were centrifuged at 4℃ and 200 g for 10 min. The cell pellet volume was suspended in the incubation medium (1% bovine serum albumin in Medium 199, with 25 mM HEPES, 2.2 g/l $NaHCO_3$, 100 IU/ml penicillin-G, 50 μg/ml streptomycin sulfate, 2550 USP K U/l heparin, pH 7.4, and treated with 95% O_2 and 5% CO_2) to 5 ml, and then added gently to the upper layer of a continuous Percoll gradient. This continuous Percoll gradient was made of adding 9 parts of Percoll to 11 parts of 1.8 × concentrated incubation medium, and then, centrifuged at 4℃ and 20,000 g for 60 min. The mixture of testicular interstitial cells was loaded onto the Percoll gradient and centrifuged at 4℃ and 800 g for 20 min.

The LC layer was collected and diluted to 10 ml in the incubation medium and then centrifuged at 4℃ and 200 g for 10 min. The LC concentration was set at 10^5 cells /ml and viability over 85-95% in this study. They were determined with a hemocytometer and the trypan blue method (Tsai *et al*., 2003). The LC culture followed the procedure described by Chen *et al*. (2002), in which purified LC was re-suspended in Medium 199 (pH= 7.4) with supplementation of 2.2 g/liter $NaHCO_3$, 2.4 g/liter HEPES, 0.1% BSA, 12.5 mg/liter gentamycin sulfate, and 0.5 mg/ml Bovine lipoprotein, and maintained at 34℃, 5% CO_2 and 95% O_2. The cultured LC was used in this study.

Two experiments were conducted to evaluate the ability of the testosterone production of the cultured LC. The first experiment measured the testosterone production of plain LC (10^5 cells / ml) incubated for 3 days with different concentrations of hCG and three steroidogenic precursors. For

the second experiment, LC was treated with hCG and 3 steroidogenetic precursors for 1, 2 and 3 days to measure the effectiveness of different time spans on the stimulation of daily testosterone production. The concentration of hCG and each of the precursors used in this experiment were determined by the first experiment.

Steroid hormone concentrations were measured with the enzyme immunoassay (EIA) (Yamauchi *et al*. 1999; Pereira *et al*. 2005); testosterone concentrations with IMMULITE 2000 assay (Siemens Medical Solutions Diagnostics Pte Ltd, 5210 Pacific Concourse Drive, Los Angeles, CA, USA) using a commercial kit (EIA L2KTW2, Simens Medical Solutions Diagnostics Pte Ltd). The Quality of Siemens Medical Solutions Diagnostics Pte Ltd was certified to ISO 13485: 2003, and the cross-reactivities of the antiserum were 100% testosterone, 2.0% 5α-dihydrotestosterone, 0.5% 5α -androstan-3β, 17β-diol, 0.6% androstenedione, 0% 5α-androstan-3,17-dione, less than 1% cholesterol and pregnenolon. Assay sensitivity was 0.2 ng/ml, and the inter- and intra- assay coefficients of variation were 5.1 and 7.2%, respectively. The IMMULITE 2000 assay was an automatic two-site sandwich immunoassay with chemiluminescent detection (Owen and Roberts 2004).

All values were given as the means ± SEM. Statistical significance between the mean values was assessed by Student's t-test with the significant level at $P < 0.05$.

Results

Experiment 1

The testosterone production by LC *in vitro* increased with the increase in concentrations of hCG and three steroidogenic precursors for all 3 deer species studied. There was a sharp increase in the production when the concentrations reached the particular levels of 0.5 IU/ml for hCG, 10^3 nM for 25-OH-cholesterol, 10^3 nM for pregnenolone, and 10^2 nM for androstenedione. Although the production continued to increase when the concentrations were over these levels, they were considered as the minimum effective concentrations and used in Experiment 2 for the sensibility enhancement experiment to differentiate the effectiveness among the treatments. Results also showed that at the same concentration, androstenedione resulted in higher testosterone production than the other two precursors (Table 1).

Experiment 2

Productions of testosterone by LC in all treatments were low and showed no significant differences on the first 2 days of the incubation, but increased significantly on the 3rd day. The quantity of the testosterone produced on the 3rd-day was higher for the 25-OH-cholesterol treatment than that of the androstenedione treatment, indicating that there was a better stimulation of testosterone production by precursors at the later stage of the steriodogenic process (Table 2).

Discussion

Seasonal changes in testosterone production of male cervids have been known to be regulated by photoperiod through the hypothalamus-pituitary-gonad axis (Sempéré *et al*. 1992; Lincoln and Kay 1979; Kameyama *et al*. 2002). It has been showed that LH is required for the maintenance of LC-specific functions, and thus, it is the main physiological factor controlling the LC's production

Table 1. Comparison[1] of the average testosterone production (nM/3 days) of cultivated Leydig cells (LC; 10^5 cells /ml) purified from hard-antlered male sambar deer, sika deer and muntjacs, and treated with various concentrations of human chorionic gonadotropin (hCG), 25-OH-cholesterol (cholesterol), pregnenolone, and androstenedione (sample size, 3 animals for each of the three species).

Treatments	Sambar deer		Sika deer		Muntjac	
	Average	SD	Average	SD	Average	SD
LC	0.76[a]	0.10	0.97[a]	0.13	0.90[a]	0.10
LC+hCG 0.05 IU/ml	0.73[a]	0.03	0.80[a]	0.06	0.87[a]	0.14
LC+hCG 0.50 IU/ml	1.46[b]	0.10	1.53[b]	0.14	2.11[b]	0.07
LC+hCG 1.00 IU/ml	1.73[c]	0.07	1.77[c]	0.03	2.22_b	0.21
LC+hCG 2.00 IU/ml	2.29[d]	0.21	2.11[d]	0.14	3.54[c]	0.10
LC	0.73[a]	0.03	0.73[a]	0.03	0.87[a]	0.07
LC+Cholesterol 10^2 nM	0.83[a]	0.14	0.76[a]	0.10	0.94[a]	0.10
LC+Cholesterol 10^3 nM	0.87[a]	0.14	0.83[a]	0.14	0.90[a]	0.10
LC+Cholesterol 10^4 nM	1.98[b]	0.24	1.94[b]	0.35	2.81[b]	0.62
LC+Cholesterol 10^5 nM	3.61[c]	0.17	2.81[b]	0.66	3.71[b]	0.38
LC	0.73[a]	0.03	0.73[a]	0.03	0.73[a]	0.07
LC+Pregnenolone 10^2 nM	0.78[a]	0.17	0.90[b]	0.14	0.94[b]	0.14
LC+Pregnenolone 10^3 nM	0.90[a]	0.14	0.90[b]	0.14	0.97[b]	0.14
LC+Pregnenolone 10^4 nM	1.87[b]	0.42	1.98[c]	0.21	3.33[c]	0.76
LC+Pregnenolone 10^5 nM	2.95[b]	1.04	3.02[c]	1.11	3.57[c]	0.87
LC	0.73[a]	0.07	0.73[a]	0.03	0.73[a]	0.07
LC+Androstenedione 10^1 nM	0.83[a]	0.14	0.90[b]	0.14	0.97[b]	0.10
LC+Androstenedione 10^2 nM	2.01[b]	0.14	2.63[c]	0.14	2.91[c]	0.14
LC+Androstenedione 10^3 nM	5.69[c]	0.17	8.36[d]	0.14	9.12[d]	0.52
LC+Androstenedione 10^4 nM	8.91[d]	0.03	9.81[e]	0.52	11.72[e]	0.28

[1] Difference in the superscript letters, a, b, c, and d indicating significant difference (t-test, $p<0.05$) in average testosterone production between two concentrations of the same treatment and species.

Table 2. Comparison[1] of daily average testosterone productions (nM) during the three-day incubation of cultivated Leydig cells (LC; 10^5 cells /ml) purified from hard-antlered male sambar deer, sika deer and muntjacs, and treated with human chorionic gonadotropin (hCG; 0.50 IU/ml), 25-OH-cholesterol (Cholesterol; 10^3 nM), pregnenolone (10^3 nM) and androstenedione (10^2 nM) (sSample size, 3 animals for each of the three species)

Treatments and incubation period (days)	Sambar deer		Sika deer		Muntjac	
	Average	SD	Average	SD	Average	SD
LC (1 day)	0.00[a]	0.00	0.00[a]	0.00	0.00[a]	0.00
LC (2 days)	0.00[a]	0.00	0.00[a]	0.00	0.00[a]	0.00
LC (3 days)	0.69[b]	0.00	0.69[b]	0.00	0.76[b]	0.03
LC+hCG (1 day)	0.69[a]	0.00	0.69[a]	0.03	0.69[a]	0.03
LC+hCG (days)	0.83[a]	0.17	0.73[a]	0.03	0.87[a]	0.17
LC+hCG (3 days)	1.39[b]	0.07	1.42[b]	0.07	1.56[b]	0.17
LC+ Cholesterol (1 day)	0.69[a]	0.03	0.73[a]	0.07	0.73[a]	0.03
LC+ Cholesterol (2 days)	0.78[b]	0.03	0.73[a]	0.03	0.76[a]	0.03
LC+ Cholesterol (3 days)	1.46[c]	0.10	1.49[b]	0.10	1.70[b]	0.10
LC+Pregnenolone (1 day)	0.69[a]	0.00	0.76[a]	0.07	0.73[a]	0.03
LC+Pregnenolone (2 days)	0.87[b]	0.14	0.83[a]	0.14	0.90[b]	0.07
LC+Pregnenolone (3 days)	3.47[c]	0.73	4.47[b]	0.94	6.24[c]	0.69
LC+Androstenedione (1 day)	0.94[a]	0.21	1.04[a]	0.00	0.94[a]	0.21
LC+Androstenedione (2 days)	1.63[b]	0.49	1.39[a]	0.35	1.80[b]	0.66
LC+Androstenedione (3 days)	18.13[c]	0.83	16.92[b]	0.87	21.95[c]	2.11

[1] Difference in the superscript letters, a, b, and c, indicating significant difference (t-test, $p<0.05$) in daily average testosterone productions between two incubation periods (days) of the same treatment and species.

of testosterone (Saez 1994; Dufau 1998).

In this study although the isolated LC spontaneously secreted small amounts of testosterone 3 days after culture, it produced more testosterone at any length of the culture with either one of hCG and three steroidogenetic precursors (Table 2). The results demonstrated that there were positive effects of the hCG and steroidogenetic precursors on the production of testosterone by deer LC. It has been found that the testosterone production of LC is enhanced with LH or its analog hCG for mice (Scott *et al*. 1989) and rats

(Ciampani *et al*.1992; Tsai *et al*. 2003; Zirkin and Chen 2000), and with steroidogenetic precursors, 25-OH-cholesterol (10^{-5} M~10^{-7} M), pregnenolone (10^{-5} M~10^{-7} M) and androstenedione (10^{-5} M~10^{-7} M) for rats (Tsai *et al*. 2003).

The results of this study also showed that testosterone biosynthesis was enhanced at greater rate by the precursors synthesized later in the steroidogenic pathway than those synthesized at the earlier stages. This was particularly true with respect to androstenedione. Two possible explanations for this phenomenon are provided herein. It was possible that the 3-day incubation period used in this study was too short to allow those products in early steroidogenic process to exhibit their effectiveness. It might be also due to the fact that androstenedione is more important precursor than the others in the biosynthetic pathway of testosterone production.

Finally, we have not only developed a useful LC system to detect androgen production *in vitro* biosynthesis for male deer, but also a technique might be useful for further understanding the functions of the LC of cervids. It might also applicable in studies of the receptor expressions of gonadotropins (LH and FSH), prolactin, and other peptide hormones, as well as of effects of drugs or pollutants on androgen production of LC of animals with reproduction seasonality.

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