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ANDROGEN/TESTOSTERONE SYNTHESIS BY THE DISSOCIATED TESTICULAR CELLS FROM MICE OF DIFFERENT AGES IN RESPONSE TO RAT LH STIMULATION *IN VITRO*[1]

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John Yuh-Lin Yu, Tsui-Ying Chang, Hseng-Kuang Hsu, Ching-Fong Liao and Walter Chia-Mo Wan (1981) Androgen/testosterone synthesis by the dissociated testicular cells from mice of different ages in response to rat LH stimulation *in vitro*. *Bull. Inst. Zool., Academia Sinica* **20**(1): 57-65. The response of the mouse testis to rat luteinizing hormone (LH) was examined for the mice at different ages using mechanically dispersed interstitial cell preparations. The cells (0.35×10^6 cells/tube) were incubated in Medium 199-xanthine-heparin medium in a Dubnoff shaker with various doses of rat LH (2-250 ng) and for different durations of time. The androgen production was measured by radioimmunoassay using testosterone antiserum. In one of the experiments, testosterone formation was also quantified after separation by celite column chromatography. The dose-response relationships and the time course patterns of androgen production during incubations were compared for the mice at 20, 40, 60, and 120 days of age. The results and conclusion may be summarized as follows: 1) On basis of the equal numbers of interstitial cells, the androgen production by the cells in response to LH was most pronounced at 40 days old, and was decreased with the advance of age; the response at 20 days of age was minimal. A dose-response relationship was obtained up to approximately 50 ng of rat LH under the conditions of the present study. 2) The numbers of total interstitial cells per animal was increased with age, as determined by microscopically counting the dispersed cell suspensions, increasing from 5.6×10^6 cells at 20 days to 40.5×10^6 cells at 120 days; androgen production based on each animal was consequently greater in the mice of 60 and 120 days old as compared to the younger mice. 3) The proportion of testosterone relative to androgens produced was essentially constant after 40 days of age, accounting for about half of total androgens formed under such *in vitro* conditions. Our study also indicates that the dispersed testicular interstitial cells from the mice between 40 and 60 days of age are highly suitable for the use of *in vitro* bioassay for LH.

The dispersed testicular interstitial cells or enriched Leydig cells have been used recently as bioassays for LH *in vitro*[5~7,11,18~23], studies on hormonal regulation of steroidogensis[4~6,13,22,25], investigations of the mechanism of LH/HCG actions[8,9], and the effects of alcohol as well as the drugs on androgen synthesis[2,26]. Such technique has also been used to compare the steroidogenic capacity for the rats at

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different developmental stages[3,13,18~20]. Among many advantages of this method, the numbers of the interstitial cells can be identified in each incubation experiment, and is thus convenient for further analysis or comparison of the parameters studied. It has been observed in the rat, using the dispersed testicular interstitial cells, that differential forms and the capacity of androgen formation were identified during maturational changes[12,18~20].

In contrast to rats, very little studies were made on the steroidogenesis of the isolated testicular interstitial cells from the mice[10,21,23]. The present study was conducted to investigate the responsiveness to LH stimulation of the dispersed interstitial cells from the testes of the mice of different ages. The time course patterns and dose-response relationships of androgen and testosterone formation were compared for the mice at 20, 40, 60, and 120 days of age by incubations of the mechanically isolated testicular interstitial cells in response to LH *in vitro*.

MATERIALS AND METHODS

Preparation of testicular interstitial cells

Mice (20, 40, 60, and 120 days old), ICR, U. S. A. were purchased from the National Laboratory Animal Resources, National Taiwan University. The animals were raised in a temperature controlled room (22±2°C), fed *ad libitum* with Purina Chow; the lighting schedule was 12L:12D.

The method in preparation of testicular interstitial cells was similar to that described previously[21] which is a modified procedure reported by Dufaud and Catt[7] and Van Dame *et al.*[23]. The animals were weighed before sacrifice by cervical dislocation. The testes were removed, weighed, and then placed in a plastic Petri dish containing 3 ml of aerated preincubation medium (Medium 199 with Hank's salts, L-glutamine and 25 mM HEPES, penicilin 10,000 units/100 ml, streptomycin 5 mg/100 ml, 0.2% bovine serum albumin, 10% sodium bicarbonate 1.0 ml/100 ml, pH 7.40). The testes were decapsulated and cut with with surgical blade into small pieces; 4-8 testes were pooled for one run. The testes were then transfered to the Erlenmyer flask which contained the preincubation medium. The testicular pieces were gently dispersed for 15 min with a magnet stirrer surrounded by an ice-bath; the medium was repeatly withdrawn into a fire-polished Pasteur pipet over several minutes until homogeneous suspension was obtained. The cell suspension was then filtered through a fine nylon mesh, and preincubated for one hour at 34°C with shaking at 25 cycles/min. The cell suspension was cooled in ice-water, and centrifuged at 6°C, 250 g for 15 min. Sedimented cells were suspended in incubation medium (preincubation medium + 0.125 mM methyl-isobutylxanthine+sodium heparin (1 ml=20,000 USP units), 0.5 ml/100 ml).

Incubations were performed in a tightly capped polyethylene tube (13×100 mm) at 34°C, shaken at 100 cycles per min in a Dubnoff incubator. The total volume in an incubation tube was 220 μl. The total numbers of interstitial cells in an incubation tube was 0.35×10^6 for all incubations throughout the entire study. The viability of the cells as determined by staining with trypan blue was approximately 70% which being similar among the various age groups. At the end of incubation (periods depended on the specific experiments as indicated in the text), the tubes were placed in ice and 2.5 ml of 0.01 M PBS, pH 7.40, was added. Tubes were centrifuged for 30 min at 6°C, 1500 g. The supernatant was collected and stored at −25°C until assays for androgen.

Radioimmunoassay of anprogen and testosterone

The androgen was determined in all experiments; in one of the experiments testosterone was also measured after chromatpgraphic separation. The radioimmunoassay procedure for androgen was a modification of Wingfield and Farner[24] and Anderson *et al.*[1]. The modified procedure quantitated total androgen, since a chromatographic separation of androgens was omitted. Briefly, 0.8 ml of the PBS diluted incubation samples was extracted with 5 ml of diethyl ether (Merck) from a freshly opened can and allowed to freeze in dry ice-ethanol

medium. The ether layer was decanted into another tube and dried under ventilation hood at 38°C. The dried residue was dissolved in 1.0 ml of 0.01 M PBS (pH 7.40) containing 0.1% gelatin and incubated at room temperature for one hour. In one experiment, aliquots of the samples were analyzed also for the testosterone using celite column chromatography[24] for separation of the steroids. Aliquots of the PBS-gelatin dissolved steroid were incubated for 20 hrs at 4°C with tritiated testosterone (1, 2, 6, 7-^{3}H-testosterone, 88.5 Curies/m mole, Amershan) and testosterone antiserum. Dextran-coated charcol was employed to separate the antibody-bound from the free steroid. Supernatant containing the bound labeled steroid was counted in a liquid scintillation counter with counting efficiency of 55%. The assay was sensitive to 10 pg of testosterone per assay tube (Fig. 1). The between-assay coefficient variation was 14.2% and the within-assay coefficient of variation was of 4.5%. Standard and incubation

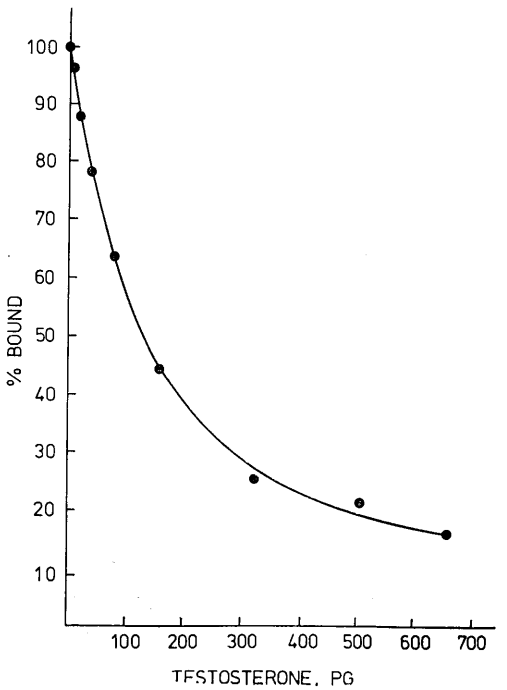

Fig. 1. Standard curve for testosterone over the range of 10-640 picogram.

samples produced parallel displacement of tritiated testosterone. The average recovery of added steroid was 91±4% (mean±SE) and individual recoveries were run with each sample.

The specificity of testosterone antiserum was described previously[1]. It cross-reacted with dihydrotestosterone, androstenedione, and androstenediol at 90-, 12-, and 11% relative to testosterone (100%), and had negligible cross-reactivities with other tested steroids. The concentration of androgen in the sample was expressed as testosterone equivalent extrapolated from the standard curve.

RESULTS

Changes of testicular weight and numbers of interstitial cells with age

The testicular weights and the numbers of total interstitial cells of various age groups are shown in Table 1. The numbers of total interstitial cells were increased with age from 20 to 120 days of age. It appears that the numbers of such cells increased only slightly after 60 days of age; the greatest change was observed to be between 40 and 60 days of age, and during this period the cell numbers was increased for 3.5 fold.

Time course patterns of androgen production by the isolated testicular interstitial cells from mice of varying age

The production of androgen from the interstitial cells, in response to rat LH (50 ng per tube), was compared among different ages of mice. As indicates in Fig. 2, androgen produced increased with time during the 6 hrs-incubation in all four age groups. The production of androgen after 4 hrs of incubation was approaching the maximal response in all age groups except that of 60 days of age where the production of androgen was still increasing until 6 hrs of incubation. The interstitial cells incubated without exogenous LH stimulation produced slight amounts of androgen during the 6 hrs incubations.

TABLE 1.

Changes in testicular weight and in the numbers and androgen synthesis capacity *in vitro* of the interstitial cells from the mice at various ages during growth[1]

Age (day)	Body weight[2] (g)	Testis weight per animal[2] (mg)	Nos. of Interstitial cells per animal[3] ($\times 10^6$)	Androgen produced at maximum response[4] (ng/0.35$\times 10^6$ cells per tube)	Maximal androgen produced by interstitial cells per animal[5] (ng)
20	10.6±0.4(9)	66±4(9)	5.7±0.4(3)	2.1±0.1(3)	33.4±0.8(3)
40	17.8±0.6(9)	104±7(9)	9.2±2.6(3)	12.9±0.2(3)	338.8±5.5(3)
60	25.1±0.9(8)	200±14(8)	32.1±7.2(3)	6.2±0.2(3)	569.5±19.3(3)
120	31.9±1.1(8)	240±10(8)	40.5±14.1(3)	3.4±0.2(3)	389.9±17.4(3)

1. All data are expressed as mean±SEM.
2. The numbers in paratheses denote the numbers of animals.
3. The numbers in parentheses denote the numbers for the preparations of interstitial cells for different incubation experiments. Each preparation consisted of pooled 4-8 testes per age group.
4. Androgen produced at maximal response as stimulated by rat LH-RP-1(NIH).
5. Androgen produced at maximal response by total interstitial cells from the testes per animal. See text for further details.

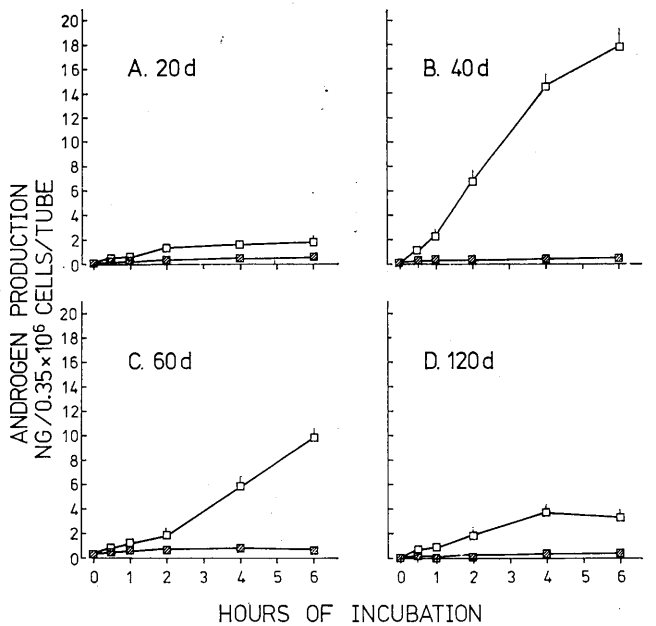

Fig. 2. Time course patterns of the response of isolated testicular interstitial cells to rat LH-RP-1 (50 ng/tube). For each incubation experiment pooled suspensions were prepared from 4-8 testes for each age group (20-, 40-, 60- and 120 days of age). Data are expressed as mean±SEM for three different incubation experiments. The vertical bars represent SEM; some of the data are expressed without SEM as the latter are smaller than the dotes. Blank squares represent androgen produced by cells incubated with LH only; squares with lines denote androgen production from cells inculated without LH.

Dose-response relationship of androgen produced by interstitial cells after exposure to rat LH in vitro

As indicated in Fig. 3, a relationship was obtained between the androgen production and varying doses of LH (up to approximately 50 ng) under the present experimental conditions. Such dose-response relationships were more pronounced for the mice of 40 days and 60 days of age as compared to those of 120 days and 20 days of age. The amount of androgen production by the interstitial cells, in response to the stimulation of LH, was decreasing after 40 days of age when the equal numbers of interstitial cells were used for incubations under identical conditions. In all four age groups the androgen production was approaching the maximum in response to rat LH of greater than 50 ng. The average androgen formation at the maximal response are shown in Table 1. The androgen produced by 40 days old mice was 6.3-, 2.1-, and 3.8 fold of the mice of 20-, 60-, and 120 days of age, respectively.

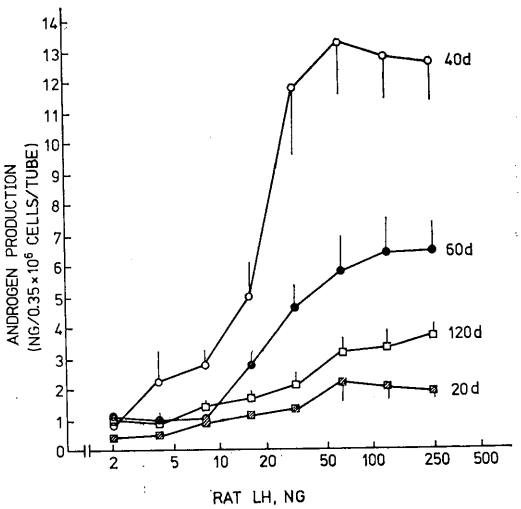

Fig. 3. Dose-response curves of isolated testicular interstial cells after exposure to rat LH-RP-1 (2-250 ng) *in vtiro*. For each incubation experiment the cell suspensions were prepared from 4-8 testes for each age group of the mice (20-, 40-, 60- and 120 days of age). Data are expressed as mean± SEM for three different incubation experiments. The vertical bars represent SEM; some of the data are expressed without SEM as the latter are smaller than the dots.

As described above, the androgen production was expressed by the same numbers of interstitial cells isolated from four age groups. However, the total numbers of interstitial cells in a testis was increased with age; as a consequence, the androgen formation capacity can be looked at by expression on a single testis or two testes of a mouse to compare the age difference. The amount of androgen produced under such *in vitro* conditions was calculated on the basis of per mouse in four age groups (Table 1). In such sense, the total amount of androgen produced by the mice was increased with age from 20 to 120 days.

Testosterone production by the interstitial cells from the mouse testes

In order to investigate the proportion of testosterone relative to the measured androgen, the production of testosterone in one incubation experiment was determined as well by further chromatographic separation for both time course patterns and dose-response relationships (Figs. 4 and 5). The amounts of testosterone production were generally parallel to those of androgen in all four age groups. The ratio of androgen over testosterone, produced by the interstitial cells following incubations with 2 to 250 ng of rat LH per tube, was in average 1.72, 1.44, 1.55, and 1.54, respectively for 20-, 40-, 60-, and 120 days of age (Fig. 5).

DISCUSSION

The results from the present study may be summarized as follows: 1) the capacity of androgen synthesis, in response to rat LH stimulation, was greatest at 40 days of age and was then decreased with age, on basis of equal numbers of interstitial cells. 2) the response was, however, more pronounced for the mice at 60 and 120 days of age on the basis of per

animal as the result of the increasing numbers of total interstitial cells in the testes with advance of the age. and 3) testosterone formation appeared to be relatively constant between 40 and 120 days of age, accounting for about half of total androgens formed.

The androgen synthesis capacity during various developmental stages have been studied in the rat recently using the dispersed testicular interstitial cells[3,16,18,20]. It has been shown that the capacity of androgen formation, in response to LH/HCG stimulation, was increased with age during pubertal maturation, studied *in vitro*[18,20]. As indicated by the present study the total androgen produced by the testis, on basis of equal numbers of interstitial cells, was most pronounced at 40 days old. A different pattern was observed when the androgen production was expressed on basis of per animal; the mice of 60 and 120 days old produced more androgen than the mice of other ages. The numbers of Leydig cells, the androgen-producing cells, relative to the total interstitial cells were not quantitatively determined in the present study. It is likely that the size of other non-steroidogenic cells was increased more rapidly than the increase of the Leydig cells during advance of the age. It has been reported in the rat studied from 10 to 110 days of age that the numbers of Leydig cells increased from 30 to 60 days and maintained the constant numbers thereafter; ^{125}I-HCG

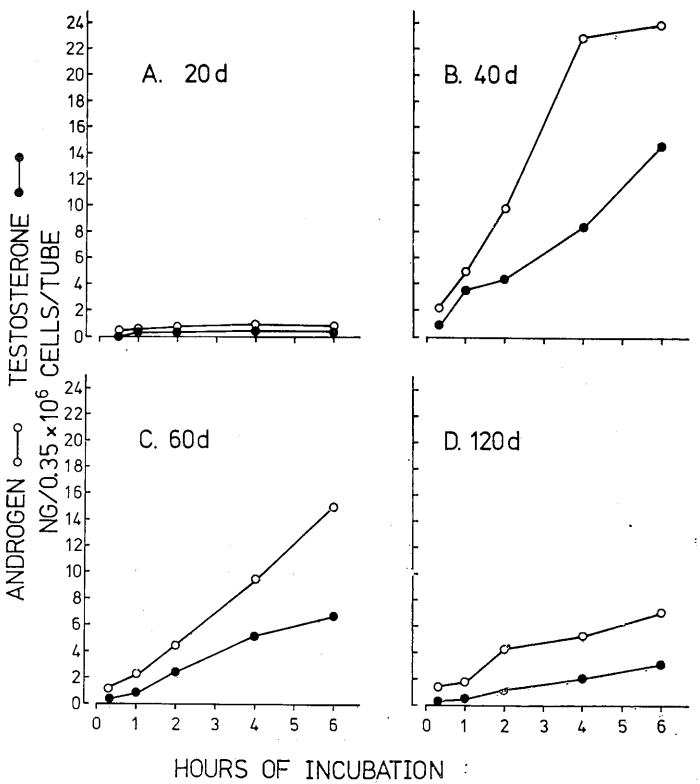

Fig. 4. A comparison of the time course patterns in the synthesis of androgen and testostrone by dispersed testicular interstitial cells in response to rat LH-RP-1 (50 ng/tube). The cell suspensions were prepared from 4-8 testes from the mice of 20-, 40-, 60-, and 120 days of age. The data represent the production of androgen and tesosterone from one incubation experiment. See Materials and Methods for further details.

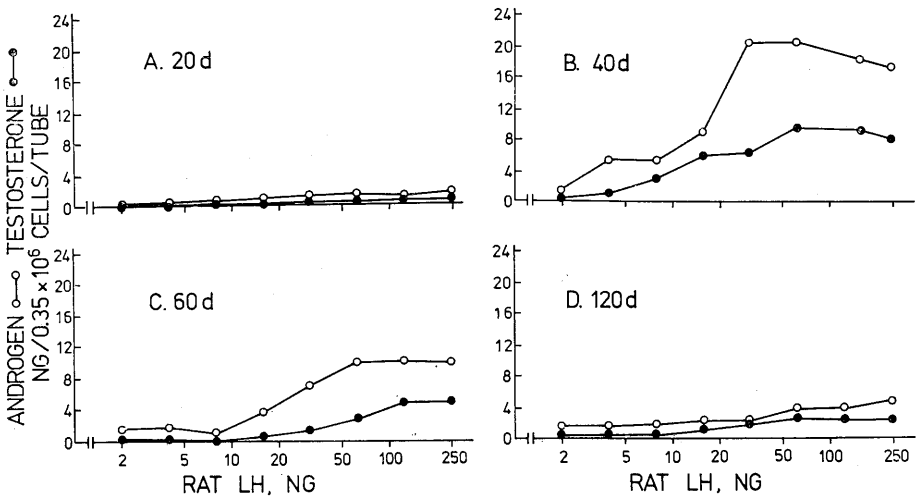

Fig. 5. Dose-response (androgen and tesosteone) curves of isolated testicular interstitial cells after exposure to rat LH-RP-1 (2-250 ng) *in vitro*. The cell suspensions were prepared from 4-8 testes from the mice of 20-, 40-, 60-, and 120 days of age. The data represent the amounts of androgen and testosterone produced from one incubation experiment.

binding capacity of Leydig cells, however, remained unchanged during the same period of time[16]. The changes in the populations of Leydig cells and the receptor binding capacity during developmental stages have not been investigated systematically in the mice. The steroidogenesis of Leydig cells, in response to LH stimulation, is complex depending on many factors such as the numbers of receptors, availability of precursors or cofactors, activities of enzymes involved in cAMP metabolism or mitochondrial steroidogenesis. The technique of incubation of interstitial cells or enriched Leydig cells, in response to hormonal stimulation *in vitro*, have thus been recently employed for investigating the problems described above in various species of animals, particularly in the rat[3,4,8,9,11~16].

In order to see the proportion of testosterone relative to total androgens formed under such *in vitro* conditions, the testosterone was also quantified in one of the experiments. It appeared that testosterone formation was essentially constant in the mice of ages between 40 and 120 days; during this period the testosterone was the major form of androgens. However, the testosterone appeared to be quantitatively minor form of the androgens at 20 days old. Such differential patterns were also demonstrated in the rat[3,17~19]. For example, it was reported that testosterone was increased from 30 to 60 days of age, and remained as a major form of androgens afterwards; while the formation of androstenediol was essentially an opposite pattern during the same age period[18,19]

The incubation of interstitial cells have also been used recently as *in vitro* biological assays for LH/HCG[5~7,11,20,23]. The mice were relatively less used as compared to the rats in this regard[17,20,23]. The results from this study provide some basic information concerning the age-related differential capacity of the mice in androgen/testosterone synthesis by isolated testicular interstitial cells. Our data thus suggest that the mice from 40 to 60 days of age highly suitable for being chosen as the animals for the *in vitro* bioassays of LH's from many mammalian species.

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小白鼠睪丸雄性素生成—比較數種年齡對精間激素之離體效應

余玉林 張翠瑛 許勝光 廖欽峯 萬家茂

本研究探討數種年齡之小白鼠，其睪丸精間細胞，在離體狀況下，對精間激素引起雄性生成之效應。精間細胞（每管含 0.35×10^6 細胞）與各劑量之大鼠精間激素 (2～250 ng) 在 Medium 199-Xanthine-Heparin 培養液中培養；產生之雄性素由放射性免疫法定量。其中有一實驗，雄性素 (Androgen) 及睪丸素 (Testosterone) 分別被測定。劑量關係與雄性素生成之時間效應，作數種年齡 (20, 40, 60 及 120 天) 之比較研究。結果指出：(1)以等數之精間細胞爲基點，睪丸雄性素生成能力以 40 天爲最大，60天及 120 天次之，20 天僅爲少量。雄性素生成與精間激素有良好劑量效應；在本試驗狀況下，50 至 250 ng 之精間激素引起雄性素之生成達到飽和。(2)睪丸精間細胞數目，隨年齡而增加；20 天爲 5.6×10^6 細胞而 120 天則爲 40.5×10^6。故如以每隻小白鼠爲單位而計算，60 及 120 天者，其睪丸雄性素生成總量較 40 及 20 天多。(3)小白鼠在 40, 60 及 120 天，其睪丸素佔雄性素之量甚爲相近，約爲 50%。本研究亦提示 40 至 60 天之小白鼠最適於作爲離體生物測定法以定量精間激素，因在此期間對雄性素生成之效應良好。