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A SENSITIVE BIOASSAY OF LUTEINIZING HORMONES FROM VARIOUS VERTEBRATE SPECIES: ANDROGEN PRODUCTION BY ROOSTER TESTIS *IN VITRO*[1]

JOHN YUH-LIN YU AND LING-MEI WANG

Endocrinology Laboratory, Institute of Zoology, Academia Sinica, Taipei, Taiwan 115, Republic of China

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John Yuh-Lin Yu and Ling-Mei Wang (1983) A sensitive bioassay of luteinizing hormones from various vertebrate species: androgen production by rooster testis *in vitro*. *Bull. Inst. Zool., Academia Sinica* **22**(1): 57-65. An *in vitro* biological assay was developed using rooster testicular slice preparations for measurement of androgenic activity of luteinizing hormone (LH). Incubations of testicular slices (5 slices/100 mg/vial), were carried out at 37°C for 4 hrs in 1.0 ml of Medium 199 containing xanthine and HEPES buffer (pH 7.40) under continuous aeration of 95% O_2—5% CO_2 in a Dubnoff incubator shaken at 100 cycles/min. Androgen of such preparations were highly responsive to LH stimulations. Dose-related androgen responses were obtained when the testicular preparations were stimulated by purified ovine or chicken LH as well as by salmon gonadotropin. Such system appears to be more sensitive than the currently available LH bioassays using avian testicular preparations, and is thus useful for *in vitro* biological assay of androgenic activity of gonadotropins from both mammalian species and certain nonmammalian vertebrates.

The development of the technique in isolating and dispersing the interstitial cells from testes has been achieved for rat, mouse, quail and turtle for *in vitro* studies of steroidogenesis in individual species *per se* or for use as bioassay of gonadotropins from heterologous or homologous species (Callard and Ryan, 1977; Dufau *et al.*, 1974; Garfink *et al.*, 1978; Maung and Follett, 1977; Ramachandran and Sairam, 1975; Van Damme *et al.*, 1974; Yu *et al.*, 1981). Among these animals, rat and mouse systems have been studied most extensively and recently have been used for the *in vitro* bioassay of LH's isolated from various mammalian species (Dufau *et al.*, 1976; Farmer *et al.*, 1977; Steiner *et al.*, 1980; Yu and Fei, 1982). Chicken testes are also responsive to mammalian gonadotropins and have been employed for *in vivo* bioassay of gonadotropins prepared from mammalian species (Breneman *et al.*, 1962).

Attempts have been made to establish the *in vitro* gonadotropin bioassay technique using dispersed tesitcular interstitial cells from chickens. Such a system would provide (1) a useful *in vitro* system for the bioassay of LH's from avian and mammalian species or even from the nonmammalian vertebrates, and (2) an assay for studying the phylogenic difference of LH molecules. Unfortunately, attempts to develop the bioassay using dispersed chicken testicular interstitial cell have been unsuccessful (Ax, 1978; Wang and Yu, 1982).

The present paper reports the establishment of an *in vitro* incubation system using rooster testicular slice preparations for bioassay of luteinizing hormones. Androgen formation capacity was used as an index for the bioassay.

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Such system is useful for *in vitro* assay of biological activity of LH's from both mammalian and nonmammalian vertebrates. It is also useful for studies of some basic aspects of steroidogenesis and its regulation in rooster testis. Some of the preliminary data was published previously (Wang and Yu, 1982).

MATERIALS AND METHODS

Animals

Five to six-months-old Dutch Hybro strain roosters (*Gallus domesticus*) were purchased from the Tongin Poultry Farm, Taipei. The chickens were raised at a temperature range of 20–25°C, the lighting schedule was 14L: 10D. The food and water were available *ad libitum*.

Hormones and chemicals

Ovine LH, NIH-LH-S22, was supplied from the National Institutes of Health, U.S.A. Chicken LH, AGCHDS-11-2312A, was a gift from Dr. S. Ishii. Salmon GTH, SG-G100, was purchased from Syndel Lab., Canada. Testosterone, xanthine (5-methyl-isobuty-xanthine), and dibutyryl cAMP (N, $O^{2'}$-dibutyryl andonosine 3′:5′-cyclic monophosphoric acid) were purchased from Sigma Chemical Co. ^{3}H-Testosterone (1, 2, 6, 7-^{3}H-Testosterone; 93.9 Curies /m mole) was from New England Nuclear. Testosterone antiserum, was a gift from Department of Laboratory Medicine, University of Washington, Seattle. Medium 199 (with Hank's salt and L-glutamine) was from Grand Island Biological Co.

Preparation and incubation of testicular slices

Standard procedure: One rooster was used in each assay. The animal was decapitated and testes (8–25 g/pair) were excised and immediately immersed into 20 ml aerated preincubation medium (Medium 199 with 25 mM HEPES buffer; 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). Testes were decapsulated, weighed, and placed into a Petri dish containing preincubation medium, and were cut into slices with surgical blade. Five slices of (about 100 mg) were pooled and weighed in a plastic Petri dish containing 2 ml medium, and then transferred into a scintillation counting vial (28×58 mm) which containing 3 ml of preincubation medium; all these procedures were carried out at room temperature (24±1°C). The vials were capped and then preincubated in a Dubnoff metabolic incubator at 37°C, shaking at 50–60 cycles/min for 60–90 min.

After preincubation was stopped, preincubation medium was pipetted out, and each vial received 0.9 ml incubation medium (preincubation medium+0.125 mM xanthine) before the LH's or gonadotropin of varying amounts (dissolved in 0.1 ml incubation medium) were added. Incubation was then performed in a Dubnoff incubator at 37°C for 4 hrs shaken at 100 cycles/min under continuous aeration. Following incubation, the vials were cooled in ice-bath and 2 ml of 0.01 M PBS was added. PBS diluted medium was transferred, centrifuged, and the superntant was collected and stored at −25°C until assay for androgen.

Establishment of optimized conditions: The effects on androgen production of variation in incubation volume (1.0; 2.0; and 4.0 ml), the numbers of testicular slices (1, 5, and 10 slices), and the length of incubation time (0.5–6.0 hrs) were compared in order to establish the optimized and validated conditions. Ovine LH-S22 was used as a standard for all the assays in the present study.

Radioimmunoassay of androgen

The redioimmunoassay procedure for androgen was described previously (Yu *et al.*, 1981). Aliquots of 0.4 ml PBS diluted incubation medium were extracted with 2.5 ml diethyl ether for 1 min. The aqueous and ether phases were separated by decanting the ether after flash-freezing the aqueous phase by immersing the bottom of tubes into ethanol-dry ice medium. Ether was evaporated under a ventilation hood in a 38°C water bath. The dried residue was dissolved in PBSG (0.01 M PBS, pH 7.40, with 0.1% gelatin) and incubated at

room temperature for one hour. The PBSG-dissolved steroid was assayed for androgen. Tritiated testosterone and testosterone antiserum were added and then incubated for 20 hrs at 4°C. 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 spectrometer. The assay was sensitive to 10 pg of testosterone per assay tube. Standard and incubation samples produced parallel displacement of tritiated testosterone.

The specificity of testosterone antiserum was described previously (Anderson *et al.*, 1975); it cross-reacted with dihydrotestosterone, androstenedione, and androstenediol at 90-, 12- and 11%, respectively, relative to tesotsterone (100%), and had negligible cross-reactivities with other test steroids. The concentration of androgen in the sample was expressed as testosterone equivalent extrapolated from the standard curve.

In some experiments, testosterone was separated from other sterois using celite column chromatography, as described by Wingfield and Farner (1975).

RESULTS

Preliminary studies on the incubations of rooster testicular slice preparations indicated that: 1) testosterone accounted for about 75% of total androgen produced by the testis following stimulation with ovine LH-S22, total androgens were thus quantified as the index of the responses to avoid the chromatographic separation of testosterone; 2) most of androgen produced from testicular slices following ovine LH stimulation was released into incubation medium while less than 20% of androgen was contained in the tissue slices; and 3) additions of 0.125 mM xanthine to the incubation medium further increased the ovine LH-stimulated androgen production (2-3 fold as compared to the controls). Xanthine was thus included in the incubation medium in the present study.

The total androgens production as stimulated by LH's, were somewhat variable between

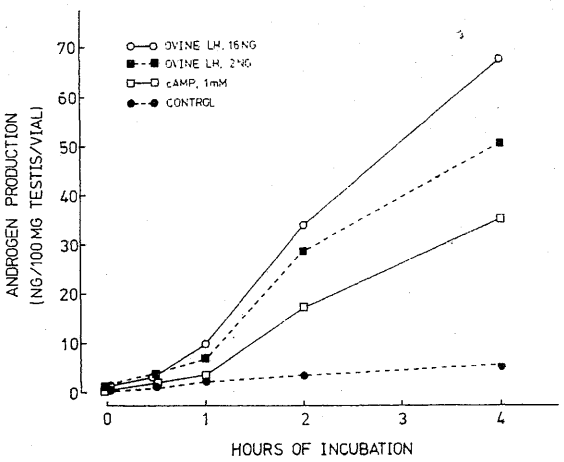

Fig. 1. Time course patterns of androgen production. Stimulation of androgen formation from chicken testicular slices (5 slices/100 mg/ml incubation medium/vial) during 4 hr-incubation with ovine LH-S22 (2 ng and 16 ng), and dibutyryl cAMP (1 mM).

different biossays. However, the % increase of androgen, relative to the controls (without LH stimulation), as stimulated by LH's, were relatively constant. Part of the data (Figs. 2, 3, and 4) on androgenic response were thus expressed as % change from the controls.

Time course patterns of androgen production

The time course patterns of androgen production by rooster testis (5 slices/100 mg/ml incubation medium) following stimulations with ovine LH-S22 and dibutyryl cAMP are indicated in Fig. 1. The control tissue (without LH stimulation) produced limited androgen during the 4-hr incubation period. The androgen formation stimulated by ovine LH increased considerably with time, being detectable as early as 0.5 hr following incubation. In a separate experiment, the time course pattern of androgen formation during 6-hr incubation period was studied with 2 ng of ovine LH-S22. Androgen formation gradually appoached the maximum level 4 hrs after incubation started. An incubation period of 4 hrs was thus chosed as the standard procedure.

Effects of variations in the size of testicular slice

In previous experiments, each incubation

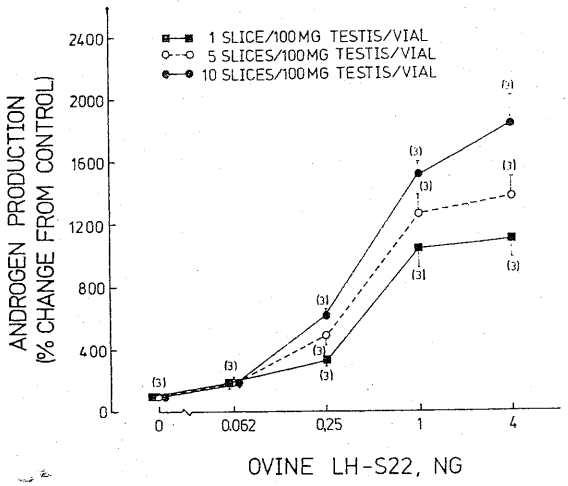

Fig. 2. Effects of variations in the size of testicular slice on androgen production. Three groups of testicular size were selected, each of them contained identical testicular weight, but varied with the numbers of slices (1 slice, 5 slices, and 10 slices/100 mg/vial). Androgen was assayed after a 4 hr-incubation of the testis/ml incubation medium/vial. The numbers in the parentheses denote the numbers of incubation experiments. The data are expressed as mean±SEM of % change (increase of androgen) relative to the controls (without LH stimulation=100%).

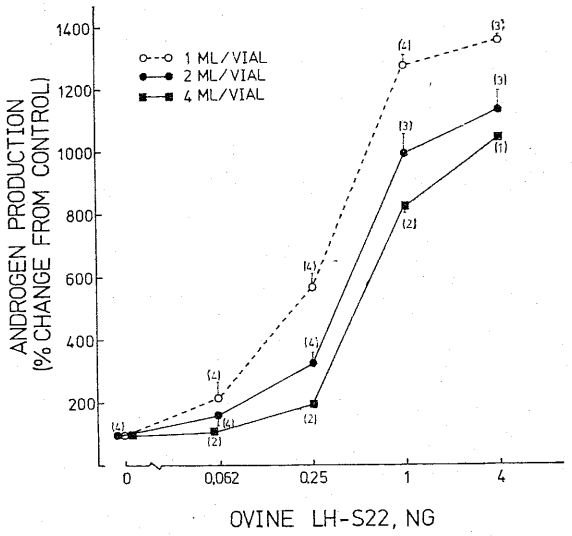

Fig. 3. Effects of volume of incubation medium on androgen production. There groups of medium volume were selected, each of them contained identical testicular weight and slice numbers (5 slices/100 mg/vial), but varied with the volume of medium (1 ml, 2 ml, and 4 ml/vial). Androgen was assayed after a 4 hr-incubation. The numbers in the parentheses denote the numbers of incubation experiments. The data are expressed as mean±SEM of % change (increase of androgen) relative to the controls (without LH stimulation=100%).

vial contained five testicular slices each weighing approximately 20 mg. In order to determine whether such size of testicular slices is optimal for androgen production, an experiment was thus conducted to compare the androgen formation capacity produced by the identical weight of testis but varying with the numbers of slices (1 slice, 5 slices, and 10 slices/100 mg/vial). As indicated in Fig. 2, at identical testicular weight, 100 mg/vial, the androgen production was most in the 10 slices/vial and was least in the 1 slice/vial, as stimulated by ovine LH-S22 ranging from 0.062 ng through 4 ng. Since the androgen produced by 5 slices/vial was only 20% less than that produced by 10 slices/vial, the former was chosed as the standard procedure in order to avoid further slicing of the testis.

Effects of the volume of incubation medium

The effect on rooster testicular androgen formation of variations in the volume of incubation medium without altering the testicular weight is shown in Fig. 3. The androgen production from 1.0 ml incubation volume was 40% and 94% greater than that from 2.0 ml and 4.0 ml incubation midia, respectively. Incubation volume below 1.0 ml was found insufficient to cover the tissues during incubation under the present experimental conditions.

Effects of aeration during incubation

An experiment was also carried out to determine whether the continuous aeration to the tissues during the 4-hr incubation period was essential for androgen production. Before incubation started, all testicular tissues received the incubation medium (1 ml/vial), that was

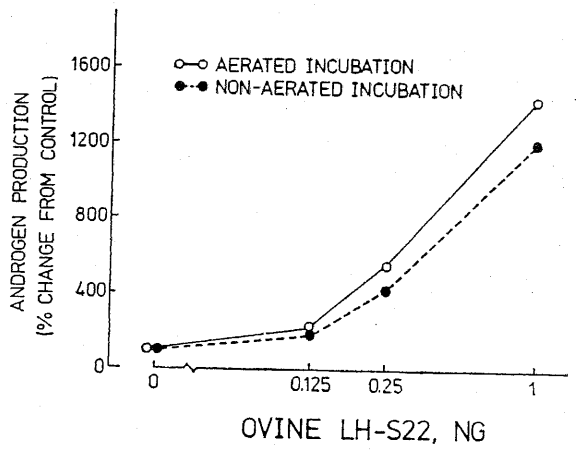

Fig. 4. The effect of aeration during incubation. For each incubation experiment (5 slices/100 mg testis/ml incubation medium/vial), one group continuously received the aeration (95% O_2—5% CO_2) during 4 hr-incubation, while the other did not (the vials being capped tightly). The data are means from two separate incubation experiments, expressed as % change (increase of androgen) relative to the controls without LH stimulation=100%).

previously aerated to a thorough extent; during the 4-hr incubation period, one group continuously received the aeration while the other did not (the vials being capped tightly). As shown in Fig. 4, the androgen production from the continuous aeration group was approximately 20% higher than that from the non-aerated group, as stimulated by ovine LH ranging from 0.125 ng through 1 ng. The supply of continuous aeration to the testicular tissues during the 4 hr-incubation did not produce a pronounced effect on androgen formation under the present experimental conditions.

Comparisons of LH's from various vertebrate species on androgen production

The androgenic activities of LH's from selected mammal, avian and piscine species were compared by the standard procedure of the rooster test system, as described in Materials and Methods. The purified LH's were incubated with 5 slices/100 mg testis/vial/ml

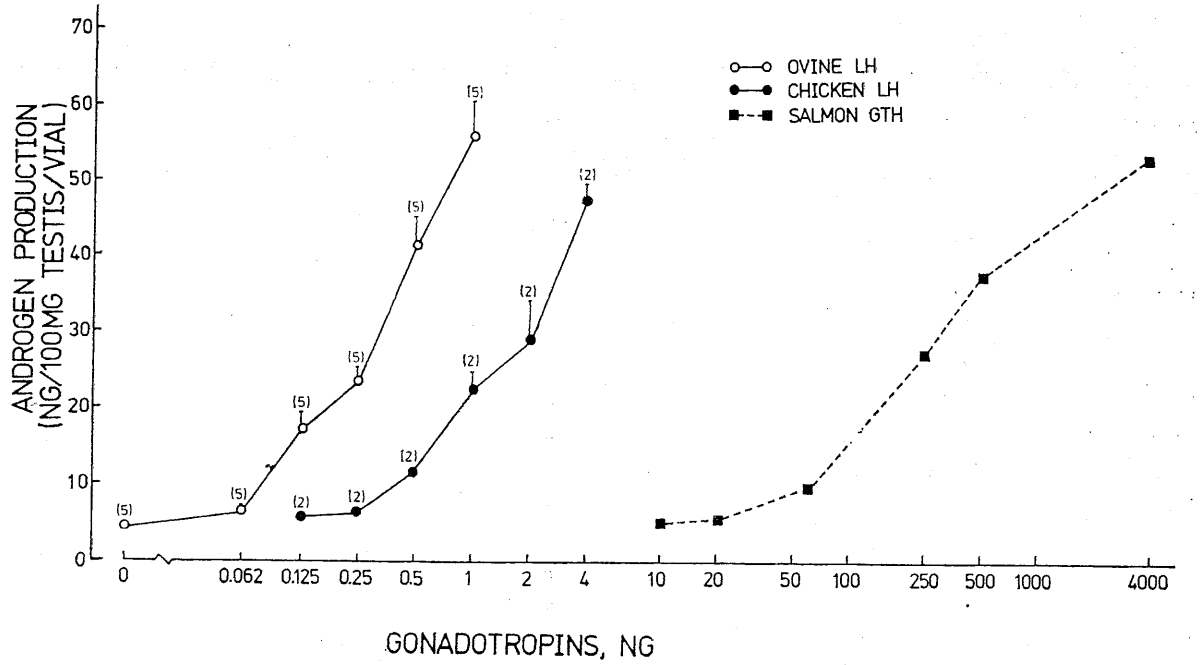

Fig. 5. The effect of selected mammalian, and avian LH's, and piscine GTH on androgen formation from incubated chicken testicular slices (5 slices/100 mg/ml incubation medium/vial). The response curves of ovine LH (0.062-1.0 ng), and chicken LH (0.125-4.0 ng) represent the results from several experiments; while that from salmon GTH (20-4000 ng) represents a single incubation experiment. The numbers in the parentheses denote the numbers of incubation experiments. The data are expressed as mean±SEM.

incubation medium, under continuous aeration at 37°C for 4 hrs. As indicated in Fig. 5, the gonadotropins from all three species elicited dose-related androgen production. The minimal doses to elicit a significant increase in androgen formation were approximately 0.1, 0.5, and 60 ng for ovine LH, chicken LH, and salmon GTH, respectively.

The mean interassay and intraassay cofficients of variation was 21.3% and 6.6%, respectively, using ovine LH-S22 (0.062; 0.125; 0.5; and 1 ng) as a standard (n=5).

DISCUSSION

The present study has established a simple and sensitive *in vitro* incubation system of rooster testicular slice preparations for illustrating the androgenic activities of gonadotropins. Such system is useful for *in vitro* bioassay of LH's from both mammalian and certain nonmammalian species, and is also suitable for studying the steroidogenesis in chicken testis *per se*.

The results of the current investigation indicate that the sliced rooster testicular preparation produced androgen in a dose-response relationship, in responding to stimulation by LH's from various vertebrate species (Fig. 5). The lower limit of the sensitivity of such system was 100 pg/assay tube when ovine LH-S22 was assayed. It was reported by other researchers that the minimal amount of ovine LH-S18 to promote androgen production was 600 pg/assay tube, using the minced rooster testis (Liu *et al.*, 1979; Glenn *et al.*, 1981). Follett and his colleagues (Maung and Follett, 1977) developed a collagenase-despersed interstitial cells system from mature quail testis and reported that the sensitivity for ovine LH-S19, on this system, was 2 ng/assay tube. Different laboratories used different LH preparations for the bioassay; this renders some inconvenience for direct comparision of the sensitivity among various assay systems used. The biological potency of ovine LH-S22 used in the present study is about 2 times of that of ovine LH-S18, and is similar to that of ovine LH-S19 assayed by ovarian ascorbic acid depletion (Licht *et al.*, 1977). It thus appears that the rooster testicular slices system developed in the present study is apparently more sensitive than the available avian test systems reported by previous workers (Ax, 1978; Glenn *et al.*, 1981; Jenkins *et al.*, 1978; Liu *et al.*, 1979; Maung and Follett, 1977).

The rooster testicular slice assay system were highly responsive to ovine and chicken LH's in terms of androgen production; it also responded to salmon gonadotropin, although the sensitivity being relatively low. Such data suggest that the rooster assay system is suitable for *in vitro* bioassay for both mammalian and avian LH's; it may also be useful for fish gonadotropin bioassay, when relatively larger amount of the hormones are available. The rat/mouse testes are higly responsive to LH's from many mammalian species; such system has thus recently been used as a convenient *in vitro* bioassay for heterologous mammalian LH's (Dufau *et al.*, 1976; Farmer *et al.*, 1977; Steiner *et al.*, 1980; Yu and Fei, 1982). However, the androgen production capabilities of such mammalian test system were considerably low in responding to avian LH's or gonadotropins from lower vertebrates (Ax, 1978; Farmer *et al.*, 1977; Licht *et al.* 1976). We have also compared the androgenic responses of LH's/GTH's in both rooster and mouse testis assays, and found that the mouse system was markedly less responsive to chicken LH's and piscine GTH's (Yu and Wang, unpublished data). It thus appears that avian testis system provides a more universal assay, than the rat/mouse system, for measuring the androgenic activities of gonadotropin molecules from various vertebrate species.

Follett and his colleagues have shown that various piscine gonadotropins stimulate androgen formation, in a dose response manner, from the isolated quail testicular cells (Jenkins *et al.*, 1978). They reported that the minimal amount of salmon gonadotropin, SG-G100, to promote androgen increase in quail system was about 2000 ng/tube. The data from the present study indicate that the sensitivity of SG-G100 to promote androgen production was as low as

60 ng/tube assayed with the rooster testicular slice system. In this regard, the chicken system provides a more sensitive assay as compared to the quail system with respect to salmon gonadotropin.

The results from the present study also reveal that ovine LH is more potent than chicken LH in promoting androgen production from rooster testis *in vitro* (Fig. 5). These findings are similar to those reported by Ax (1978), using *in vitro* conditions. From the phylogenic point of view, one would expect the avian LH to be more potent than mammalian LH in promoting androgen production from rooster testis. It was demonstrated that the crude anterior pituitary preparations from chickens were superior to purified mammalian LH in eliciting the biological effect in chicken studied under *in vivo* conditions (Ax, 1978). Such findings would suggest that the reduction of biological activity of avian gonadotropins may have occurred during purification and isolation procedures; and thus led to the decreased potency of the avian gonadotropins *in vitro*.

The development of the technique in isolation of the interstitial cells or Leydig cells from testes have been achieved for various mammalian and nonmammalian vertebrates, for bioassays of LH's/GTH's from homologous or heterologous species (Callard and Ryan, 1977; Dufau *et al.*, 1974; Dufau *et al.*, 1976; Farmer *et al.*, 1977; Garfink *et al.*, 1978; Jenkins *et al.*, 1978; Maung and Follett, 1977; Ramachandran and Sairam, 1975; Steiner *et al.*, 1980; Van Damme *et al.*, Yu *et al.*, 1981; Yu and Fei, 1982). Such system has, at least, two advantages: firstly, the steroidogenic cells can be identified and the numbers of the cells can be controlled in the studies; and secondly, the contact between the cells and the stimulants are maximal resulting in the best sensitives as compared to the slices or minced testes. Attempts to develop the parallel technique were, however, unsuccessful, by far, for the chicken testes (Ax, 1978; Wang and Yu, 1982). It was found that the dispersed interstitial cells from the rooster testes were viable as evidenced by dye uptake technique; these cells were not capable in production of androgen in response to LH stimulations (Wang and Yu, 1982). As an alternative, preparations and incubations of testicular slices from the roosters were thus developed for the use of *in vitro* bioassays of LH's/GTH from various vertebrate species. Further efforts devoted to establish the isolated interstitial cell system from rooster tester are thus required.

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多種脊椎動物黃體生成激素之生物測定：離體雞睾丸組織雄性素之生成

余 玉 林　　王 玲 美

本研究之目的有二：(1)建立鷄睾丸組織雄性素生成之最適宜之離體培養系統；(2)利用此一離體培養系統、進行黃體生成繳素 (luteinizing hormone, LH)，或促性腺素 (gonadotropin, GTH) 之離體生物測定。

離體培養狀況之試驗：將睾丸組織，黃體生成繳素，與培養液 (Medium 199-Xanthine, pH 7.40) 一起培養於 37°C 水浴中，測定各種培養情況對黃體生成繳素促進睾丸雄性素生成之影響——不同培養時間 (0.5～6 hr)，睾丸組織切片之大小 (1, 5, and 10 slices/100 mg/vial)，培養液之體積 (1, 2, and 4 ml)，以及培養期間連續供應混合氣 (95% O_2－5% CO_2)。試驗結果綜合出最適宜之離體培養狀況爲 5 slices/100 mg testis/ml incubation medium/vial，並且需連續通以混合氣，在 37°C 培養 4 小時。

各種黃體生成繳素或促性腺素之生物測定：羊與雞之黃體生成繳素，以及鮭魚之促性腺素 (Ovine LH-S22; Chicken LH-AGCHDS-11-2312A; Salmon GTH-SG-G100)，均能促進雄性素之生成，且有良好之劑重相關；其最低敏感度分別 0.1, 0.5, 與 60 ng。此培養系統之敏感度高於其他以鳥類睾丸所進行之黃體生成繳素/促性腺素之離體生物測定法；同時，可做爲哺乳類，鳥類及魚類之促性腺素之離體生物測定，因而比哺乳類之睾丸測定系統有更廣泛之應用性。