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# STEROIDOGENESIS IN THECA CELLS OF CHICKEN FOLLICLES

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## ABSTRACT
The aim of this study was to assess and compare the steroidogenic response to luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in cells of the theca interna and theca externa isolated from follicles at different stages of maturation. Cells of the theca interna and theca externa isolated from the largest, second largest and third-fifth largest preovulatory follicles of the chicken (*Gallus domesticus*) were incubated at the concentration of 2 x $10^5$ cells/ml/tube in Medium 199 containing 10mM Hepes and 0.2% bovine serum albumin with increasing doses (0, 0.1, 1.0, 10 and 100 ng/ml/tube) of ovine LH (oLH) or ovine FSH (oFSH). After the 4 hr-incubation, progesterone ($P_4$), testosterone (T), and estradiol-17 β ($E_2$) in the medium were measured by RIA. $P_4$ and T were detected in cultured medium of theca interna cells but were undetectable (< 25pg/tube) in that of theca externa cells, while $E_2$ was detected in cultured medium of theca externa cells but not (< 25 pg/tube) in that of theca interna cells. The productions of P4 and T were stimulated in a dose dependent manner by oLH and oFSH. The responses to oLH and oFSH were greater in smaller follicles, although the response to oFSH was less than that to oLH. $E_2$ production was stimulated in a dose-dependent manner by oFSH with greater response in smaller follicles but not by oLH. The present results support a three-cell theory for estrogen production in avian follicles and indicate that, in the chicken, the smaller follicles may play a conspicuous role in gonadotropin-induced steroidogenesis.

## KEYWORDS
Chicken, gonadotropin, steroidogenesis, theca interna and externa cells.

## RESUMEN

El propósito de este estudio fue de ensayar y comparar la respuesta esteroidogenica a la hormona luteinizante (LH) y la hormona del folículo estimulante en las células de la teca interna y externa, aisladas desde folículos en diferentes estados de maduración. Las células de la teca interna y externa aisladas de primeros largos y segundo y tercero-quinto largos folículos preovulatorios de la gallina (*Gallus domesticus*), fueron incubados a concentración de 2 $X10^5$ células/ml/tubo, en medio 199 conteniendo 10mM Hepes y 0.2% de suero de albumina de bovino, con incremento de dosis (0, 0.1, 1.0, 10 y 100 ng/ml/tubo), de ovino LH (oLH) o ovinoFSH (oFSH). Después de 4 horas de incubación, progesterona ($P_4$) Testosterona (T), y estradiol 17ß ($E_2$), fueron medidos por RIA, $P_4$ y T fueron detectados en el medio de cultivo de la teca interna, pero indetectable (< 25 pg/tubo) en células de teca externa, mientras que $E_2$ fue detectado en medios de cultivo de células de la teca externa, pero no (< 25 pg/tubo), en células de la teca interna. La producción de $P_4$ y T fue estímulado en dosis dependiente por oLH y oFSH. La respuesta a oLH y oFSH fue grande el folículos menores, aunque la respuesta a oFSH fue menor que oLH. La producción de estradiol fue estimulada en dosis dependiente por oFSH, con gran respuesta en pequeños folículos pero no por oLH. El presente resultado soporta la teoría de tres células para la producción de esteroides en folículos de ave e indica que en la gallina, los pequeños folículos juegan un papel conspicuo en que la gonadotropinas-induce la esteroidogenesis.

## PALABRAS CLAVES

Gallina, gonadotropina, steroidogenesis, células de teca interna y externa.

## INTRODUCTION

In avian, the major hormone being secreted from the ovarian follicle are progesterone ($P_4$), testosterone (T) and estradiol17-β ($E_2$) (Gilbert, 1977). It has well been established that, with follicular maturation, there is a marked increase in steroidogenic activity of the granulosa cells which produce mainly $P_4$ whereas steroidogenic activity in the theca cells, which produce T and $E_2$, is decreased (Shahabi et al., 1975; Huang & Nalbandov, 1979; Nakamura et al., 1979; Bahr et al., 1983; Marrone & Hartelendy, 1983). A three-cell theory for estrogen production in avian species has recently been proposed (Porter et al., 1989; Pedernera et al., 1989; Nitta et al., 1991; Velazquez et al., 1991; Kato et al.,1995, Caicedo, 1997); this theory is based on the facts that at least two kinds of steroidogenic cells are present in the theca layer of preovulatory follicles, and $P_4$ produced by granulosa cells is converted by theca interna cells to androgens, which is subsequently metabolized to estrogen by theca externa cells. The production of $P_4$

in granulosa cells is stimulated by gonadotropins, lutheinizing hormone (LH) and follicle-stimulating hormone (FSH). As the follicles mature, the granulosa cells sensitivity to LH is increased while the sensitivity to FSH is decreased; by the last day before ovulation, the steroidogenic response to FSH disappears and the granulosa cells solely under the regulatory influence of LH (Huang et al., 1979; Scanes & Fagioli, 1980; Hammond et al., 1981; Robinson et al., 1988). In contrast to the granulosa cells, the role gonadotropins in the control of steroidogenesis in cells of the theca interna and theca externa of chicken preovulatory follicles has not been clearly defined, although the production of T and $E_2$ in the theca cells of avian follicles has been shown to be influenced by gonadotropins hormone (Huang & Nalbandov, 1979; Onagbesan & Peddie, 1989; Porter et al., 1989, 1991; Nitta et al., 1991).

The purpose of the present studies was to evaluate and compare the steroidogenic activity in response to gonadotropins hormone, LH and FSH, in cells of the theca interna and theca externa isolated from follicles at different stages of maturation.

## MATERIALS AND METHODS

### Animals:

White Leghorn hens laying at least four eggs in a sequence were used. Seven to ten hen 1-2 hr after oviposition were killed for each of at least three replications, and the largest ($F_1$), second largest ($F_2$) and third-fifth($F_3$-$F_5$) largest preovulatory follicles were removed from the ovary of each hen.

### Isolation of theca interna and theca externa cells

After removing the outer fibrous tissues and the separation of the granulosa layer according to the method of Gilbert et al. (1977), the remainder of each follicle (*theca folliculi*) was inverted, and incubated for 30 min in medium 199 containing 10mM Hepes and 0.2% collagenase. The theca interna layer was gently scraped off with a scalpel blade until the color of tissue changed from pink to near white, and returned to Medium 199 containing 10mM Hepes and 0.2% collagenase, then further incubated for 20 min. after the incubation, mechanical dispersion was performed for 5 min with a syringe. The remaining theca externa layer was minced into about 2 mm square

pieces, and incubated for 60 min in Medium 199 containing 10mM Hepes and 0.2% collagenase with mechanical disruption of tissue at 15 min interval with a syringe. The cell suspensions of theca interna and theca externa were filtered through a nylon gauze (60µm mesh) and centrifuged at 400 X g for 10 min at 4°C. The cell pellet was resuspended in Medium 199 containing 40% Percoll and centrifuged at 400 X g for 20 min. Top layer containing theca cells was removed and pelleted by centrifugation at 400 X g for 10 min. The cell pellet was suspended in culture medium containing 10mM Hepes and 0.3% bovine serum albumin (BSA) in Medium 199, and centrifugated at 400 X g 10 min. This cell-washing procedure was repeated three times.

**Culture of theca cells**

After the cell count on a hemocytometer following a trypan blue dye exclusion, living cells of the theca interna and theca externa were diluted to 2 X $10^5$ cells/0.5 ml with culture medium. oLH (NIDDK oLH-S26) and oFSH (NIDDK oFSH-S20) were diluted in culture medium at the concentrations of 0.1, 1, 10 and 100 ng/0.5 ml and added to the cell suspensions. The cells with the incubation volume of 1ml were incubated for 4 hr at 37°C. after the incubation, the cultured medium was stored –20°C until assayed for steroid hormones.

**Assays of steroid hormones**

$P_4$, T, and $E_2$ in the medium were measured in duplicate without extraction by a routine radioimmunoassay. The antiserum of $P_4$, T and $E_2$ were donated by Dr. Wakabayashi (Gunma University, Japan). Radioactive steroids [1, 2, 6, 7 - $^3$H] $P_4$, [1, 2, 6, 7 -$^3$H] T and [2, 4, 6, 7, 16, 17- $^3$H] $E_2$, were purchased from Amersham Company. In brief, in glass tubes (10 X 75 mm), 0.1ml of the cultured medium diluted with 1% BSA-0.1M phosphate buffered saline, 0.1 ml of $P_4$, T or $E_2$ anti-serum diluted with 1% normal rabbit serum-0.05 M EDTA-0.1M phosphate buffered saline and 0.1 ml of 1% BSA-0.1M phosphate buffered saline containing $^3$H-$P_4$, $^3$H-T or $^3$H-$E_2$ of about 20,000-25,000 cpm were added, and incubated for 24 hr at 4ºC. For the separation of bound and unbound steroids, 0,2ml of dextran-coated charcoal suspension (6.5g charcoal Nori A and 0.625g dextran T-70 per liter of 0.01M phosphate saline) was added, and the tubes were kept in an ice bath for 30 min. After the centrifugation at 3,000 rpm for 15 min at 4ºC, the supernatant was added to a vial containing about 4 ml of ASC-II scintillator and vortexed for 10 sec. On the following

day, the radioactivity was counted for 5 min. The assay sensitivity of $P_4$, T and $E_2$ (more than 2 SD difference from zero bound) was 2.5 pg per tube in all steroids. The intra-and inter-assay coefficients of variation of $P_4$, T and $E_2$ were 6.0 and 12.5, 10.2 and 15.2 and 8.5 and 11.2% respectively.

**Statistical analysis**

The data were analyzed by analysis of variance and followed by Duncan's new multiple range test to determine significant difference among treatment means.

## RESULTS

In a few cases of the present experiments, small amounts of $P_4$, T, $E_2$ were detected in the cultured media of theca externa cell and theca interna cells, respectively. However, in most experiments $E_2$ was not detected (P<25 pg/tube) in the cultured medium of theca interna cells while $P_4$ and T were undetectable in the culture medium of theca externa cells. Therefore, because the detections of $E_2$ in theca interna cells and of $P_4$ and T in theca externa cells, the data obtained in the experiment which the separation of thecal cells was deemed to be incomplete, were thus eliminated from the present results.

The results of the measurement for $P_4$, T and $E_2$ in the cultured medium are shown in Figs. 1, 2, and 3, respectively. As shown in Figs, 1 and 2, $P_4$ and T were detected in the cultured medium of theca interna cells but below the measurement limit for these hormones (< 25 pg/tube) in the cultured medium of theca externa cells. $E_2$, however, was detected in the cultured medium of theca externa cells but not in the cultured medium of theca interna cells (Fig.3). Basal productions of $P_4$ and T by cells of the theca interna and of $E_2$ by cells of the theca externa, determined in untreated cells during the 4 hr incubation period, were 44.3-64, 232- 450 and 113-151 pg/2X$10^5$ cells/4 hr in the cells of $F_1$, 110-124, 280-326 and 254- 482 pg/2X$10^5$ cells / 4hr in the cells of $F_2$, and 130-136, 222-4429 and 352-483 pg/2 X$10^5$ cells/4hr in the cells of $F_{3-5}$ respectively. The production of $P_4$ and T by cells of theca interna was stimulated by both oLH and oFSH in a dose-related manner. However, the extent of the stimulation was different not only between the treatments of oLH and oFSH but also among follicles with different stage of development. Thus, the response to oLH in both production of $P_4$ and T was greater

than the response to oFSH. Moreover, there was a greater response in smaller follicles. The production of $E_2$ by cells of the theca externa was stimulated only by oFSH in a dose-dependent manner, but not by oLH. Also the $E_2$ production in response to oFSH was greater in smaller follicles.

## DISCUSSION

The present studies, confirm a three-cell theory for estrogen production by preovulatory follicles of the domestic hen in which at least two thecal cells subpopulations are involved in estrogen production in the theca layer; the theca interna cells produce androgens, which are subsequently metabolized to estrogen by theca externa cells (Porter et al., 1989: Pedernera et al., 1989; Nitta et al., 1991; Velazquez et al., 1991; Kato et al., 1995; Rodriguez-Maldonado et al., 1996), because $P_4$ and T were detected in cells of the theca interna but not in cells of theca externa, while $E_2$ was detected in cells of the theca externa but not in those of the interna. In addition, the presents study provides a profile of steroidogenesis in response to LH and FSH in cells of the theca interna and theca externa during the development of preovulatory follicles of the chicken.

As has been already known by the presence of cytochrome $P_{450}$ cholesterol side chain cleavage (Kowalsky et al., 1991; Nitta et al., 1991) and 3β-hydroxysteriod dehydrogenase (Nitta et al., 1993) in the theca interna, the present study confirm that $P_4$ is produced in theca interna cells, although the production of $P_4$ is very little compared to that in cultures of granulosa cells reported by many investigators (Johnson, 1990). In addition, the present study demonstrated that theca interna cell production of $P_4$ with T in all follicles examined is stimulated by not only LH but also FSH, although less by FSH than LH. Moreover, the theca interna cell response to these gonadotropins was greater in smaller preovulatory follicles. To the best of our knowledge**,** only Porter et al. (1989) had examined the effect of both LH and FSH on steroidogenesis by theca interna cells. They reported that in the turkey, the production of $P_4$ and androgen in theca interna cells was stimulated by oLH in the smaller $F_5$ but not in the larger $F_1$ and also not by porcine FSH at the concentration of 1,000 ng/ml in both $F_1$ and $F_5$. Although the distinctions between our and their results on the response of theca interna cells to LH and FSH are unclear, they used trypsin while we

used collagenase for the isolation of theca cells. As the damage to cells by enzymatical dispersion is generally known to be greater by trypsin than by collagenase, the difference in enzyme used may explain the discrepancy for the theca interna cell response to gonadotropins between our and their results. The presence of receptors for LH and FSH in avian theca layer has been reported by Kikuchi & Ishii (1992) & Masuda et al. (1984), respectively, who found that these receptors are more numerous in amaller than larger follicles. Therefore, the higher sensitivity of theca interna cells to gonadotropins in smaller preovulatory follicles may be attributed to the greater number of gonadotropin receptors in smaller follicles.

In the present experiments, the production of $E_2$ in theca externa cells was not stimulated by LH. The present results are in agreement with those of Porter et al. (1989) and Nitta et al. (1991), who also demonstrated that estrogen production is stimulated by the coincubation of theca interna and theca externa cells and enhanced in the presence of oLH. Porter et al. (1989) also reported that $E_2$ production by theca externa cells of $F_5$ was not enhanced by oLH in spite of the addition of T ($10^{-7}$ or $10^{-6}$ M) as substrate of estrogen, whereas $E_2$ production by the addition of $10^{-6}$ M of T was greater than with the addition of $10^{-7}$ M of T. The present results, together with all of the above mentioned results, indicate that LH does not stimulate the activity of aromatase which converts androgens into estrogens. However, we found that FSH, with its greater response in smaller follicles, enhances the production of $E_2$ in cultures of theca externa cells alone despite the undetectable level of T as a precursor of estrogens. Nitta et al. (1991), had reported that fibroblast in the theca externa may function as an additional site of conversion of $P_4$ to androtenedione as a substrate to produce estrogens. Therefore, the increase in $E_2$ production by FSH in cultures of theca externa cells alone indicates that FSH may enhance the aromatase activity in cells of the theca externa of the chicken.

As described above, the activity for steroidogenesis in avian theca layer is greater in smaller preovulatory follicles, so the steroidogenic capability in the theca layer is decreased with follicular development. This decrease has been explained by the decrease in the enzyme activity for steroidogenesis (Nakamura et al., 1979; Marrone & Hertelendy, 1985, Mori et al., 1985; Nitta et al., 1993), the responsiveness to LH (Porter et al., 1991), the number of receptors for gonadotropins (Masuda

et al., 1984; Kikuchi & Ishii, 1992), the mRNA levels of cytochrome P450 $_{17\alpha \text{ hydroxylase}}$ and P450 $_{\text{aromatase}}$ (Kato et al., 1995) in the theca layer, and by the interaction of the granulosa and theca layers (Huang & Nalbandov, 1979; Marrone & Hertelendy, 1983; Mori et al., 1984; Johnson et al., 1987). Based on the responsiveness of isolated theca interna or theca externa cells to LH and FSH for steroidogenesis, we conclude that the smaller preovulatory follicles exert a profound influence on gonadotropin-induced steroidogenesis in the theca layer of the chicken. Also exert a very important influence in the production of steroid hormone in ovarian development follicles.

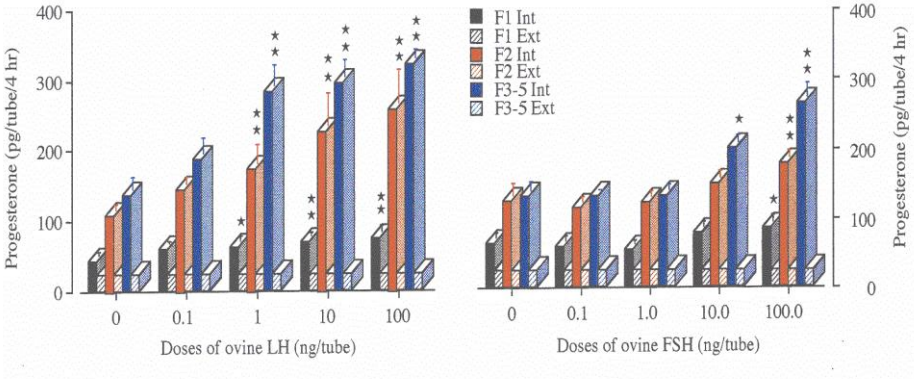


Fig.1. Effect of ovine LH and FSH on progesterone by cells of theca interna and theca externa, isolated from the largest, second largest and third-fifth preovulatory follicles. Cells of theca interna (Int), theca externa (Ext), isolated from the largest ($F_1$), second largest ($F_2$) and third-fifth ($F_{3-5}$) largest preovulatory follicles were incubated for 4 hr with or without increasing of doses of (0.1-100 ng/tube) of ovine LH or FSH. After the incubation, progesterone in the médium was measured. Results are expressed as mean±SEM of at least 3 replicates. Asterisks indicate significantly a difference (***P < 0.05, ** P < 0.01)**, from basal production (Without ovine LH and FSH) of each follicle.

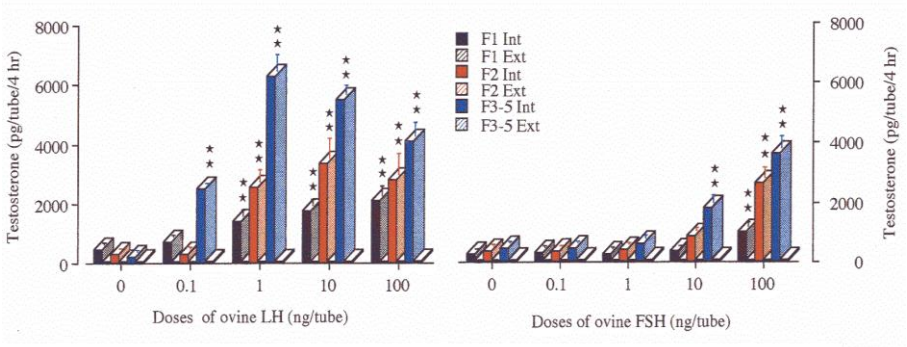


Fig. 2. Effect of ovine LH and FSH on testosterone by cells of theca interna and theca externa from the largest, second largest and third-fifth preovulatory follicles. Cells of theca interna (Int), theca externa (Ext) from the largest ($F_1$), second largest ($F_2$) and third-fifth ($F_{3-5}$) largest preovulatory follicles were incubated for 4 hr with or without increasing of doses of (0.1-100 ng/tube) of ovine LH or FSH. After the incubation, testosterone in the medium was measured. Results are expressed as mean ± SEM of at least 3 replicates. Asterisks indicate significantly a difference **(* P < 0.05, ** P < 0.01**) from basal production (without ovine LH and FSH) of each follicle.

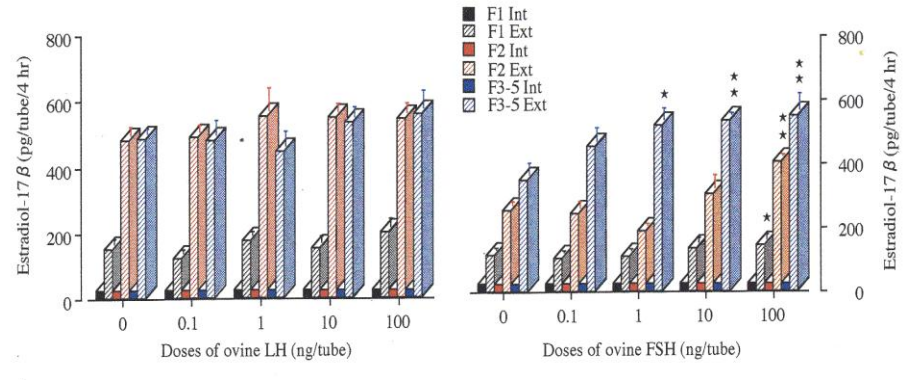


Fig. 3. Effect of ovine LH and FSH on estradiol-17ß by cells of theca interna and theca externa isolated from the largest, second largest and third-fifth preovularory follicles. Cell of theca interna (Int), theca externa (Ext), isolated from the largest ($F_1$), second largest ($F_2$) and third-fifth ($F_{3-5}$) largest preovulatory follicles were incubated for 4 hr with or without increassing of doses of (0.1 -100 ng/tube) of ovine LH or FSH. After the incubation, estradiol-17ß in the medium was measured. Results are expressed as mean ± SEM of at least 3 replicates. Asterisks indicate significantly a difference **(* P < 0.05, ** P < 0.01**) from basal production (withouth ovine LH and FSH) of each follicle.

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