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# THE INFLUENCE OF FSH, LH AND TESTOSTERONE ON STEROIDSECRETION BY TWO SUBPOPULATIONS OF PORCINE GRANULOSA CELLS

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The first objective of this research was to define the capacity of two types granulosa cells (mural MGs and antral AGc) to secrete estradiol ($E_2$) in the presence of an androgen substrate (testosterone).

The second objective was to identify the synergism between gonadotropic hormones such as LH and FSH and testosterone (T) as measured by $E_2$ secretion by MGc and AGc. MGc were separated from AGc by filtration through a 32.5 µm nylon filter. In the first experiment, control cultures were grown in M199 medium supplemented with 10% calf serum while the other cultures were supplemented with either T alone at a concentration of $1 \times 10^{-7}$ M or with $1.0 \times 10^{-3}$ M, $0.5 \times 10^{-3}$ M, or $0.1 \times 10^{-3}$ M aromatase inhibitor CGS 16949A plus T.

In the second experiment, cultures were enriched either with $1 \times 10^{-7}$ M T or with 100 ng LH plus T or 100 ng FSH plus T. After 24 hours of cultivation all cultures were terminated and the media were frozen at $-20°C$ for further steroid analysis. Basal $E_2$ secretion was always higher in MGc than in AGc. Adding testosterone to the culture medium decreased $E_2$ secretion by MGc but significantly increased $E_2$ secretion by AGc. LH in the absence of T was without effect on $E_2$ secretion by both Gc types. FSH alone stimulated $E_2$ secretion by MGc and decreased $E_2$ secretion by AGc. Synergistic action of FSH and LH with T on $E_2$ secretion by both types of granulosa cells was observed. These findings provide the basis for further analysis of the differences in aromatizing ability of the two subpopulations of Gc during follicular development.

K e y   w o r d s: *antral granulosa cells, mural granulosa cells, gonadotropic hormones, Estradiol, Testosterone*

## INTRODUCTION

The granulosa cells of the preovulatory follicle are comprised of two populations of cells: mural Gc lining the follicular wall and antral Gc surrounding the antrum. Several studies have shown morphological and physiological heterogeneity in Gc. Kasson *et al.* (1) demonstrated that fractions

of Gc have differential responsiveness to FSH and VIP. Rao *et al.* (2) suggested functional heterogeneity of Gc populations obtained from immature rats injected with diethylstilbestrol (DES) and PMSG in their response to FSH-induced steroidogenesis. Various autoradiographic studies have demonstrated differential localization of cytochrome $P_{450}$ (3), LH/hCG receptors (4, 5) and 3β HSD (activity (6, 7).

In the first series of studies MGc and AGc isolated from large preovulatory follicles were separated and their ability to aromatase exogenous testosterone was determined. In addition, studies were done on the influence of adding aromatase inhibitor to the culture medium on $E_2$ secretion by both types of Gc. The second series was intended to determine the influence of the gonadotropic hormones LH and FSH on aromatase activity.

## MATERIALS AND METHODS

### Chemicals

* M199 medium, PBS and calf serum (Laboratory of Sera and Vaccines, Lublin, Poland).
* Testosterone (Sigma).
* Aromatase inhibitor CGS 16949 (4, 5, 6, 7, 8-tetrahydroimidazol[1, 4-alpydrin-5-yl] benzonitryle) monohydrochloride (Ciba-Geigy GA, Basel).
* FSH/NIH/FSH-S-8 ovine, LH/NIH/LH-S-17 ovine (donated by the National Pituitary Agency of NIH, Bethesda, MD, USA).

### Methods

Porcine ovaries were obtained at a local slaughterhouse. The stages of the estrus cycle were determined using the criteria described by Chaning and Ledwitz-Rigby (8). Proestrus ovaries containing large (0.8—1.2 cm external diameter) vascular follicles and either no corpora lutea or corpora lutea albicantia only were selected as the source of tissue for culture. Granulosa cells were isolated by the technique described by Stokłosowa *et al.* (9). The Gc were washed twice with PBS and filtered through a 0.32 um nylon filter (NEWARK WIRE CLOTH CO., NEWARK, NJ, USA). Cells that passed through the filter were designated as antral granulosa cells (AGc). Retained material included oocytes and agregates of mural granulosa cells (MGc). Both antral and mural Gc were centrifuged for 10 min at 1000 × g and the pellet was resuspended in M199 medium supplemented with 10% calf serum and penicillin (100 U/ml). For all experiments the average concentration of MGc was 750.000 ± 250.000 cells/ml and that of AGc was 1.250.000 ± 250.000 cells/ml.

### Experiment 1

To determine MGc and AGc response to testosterone, cells were cultured with testosterone in a concentration $1 \times 10^{-7}$ M. One hour after establishment of cultures, $0.1 \times 10^{-3}$ M, $0.5 \times 10^{-3}$ M or $1 \times 10^{-3}$ M aromatase inhibitor CGS 16949 A were added for the next 23 hours in a humidified atmosphere of 5% $CO_2$ in air (all in triplicate). All cultures were terminated 24 hours after establishement and the media were frozen at $-20°C$ for further $E_2$ analysis.

## Experiment 2

To determine the influence of gonadotrophic hormones (LH, FSH) on the aromatization capacity of both MGc and AGc, the cells were grown in medium supplemented with $1 \times 10^{-7}$ M testosterone or 100 ng LH plus $1 \times 10^{-7}$ M testosterone or 100 ng FSH plus $1 \times 10^{-7}$ M testosterone (all in triplicate). After 24 hours all cultures were terminated and the media were frozen at $-20°C$ for further $E_2$ analysis.

## STEROID ANALYSIS

Estradiol was estimated according to a method described elsewhere [10]. Antiserum generated in rabbit against estradiol-17β-6-oxine BSA was used. The specificity of the antiserum for estrogen was high. It cross-reacted with estradiol (100%) and cross-reacted significantly with estrone and estriol (66% and 2.1% respectively). A series of the other steroids were tested for cross-reactivity and found to cross-react less than 0.01%. The limit of assay sensitivity was 5 pg. The coefficients of variations within and between assays were less than 7.5% and 8% respectively.

## STATISTICAL ANALYSIS

Significant differences between the concentrations of steroids in the control and experimental cultures were compared by analysis of variance and Duncan's new multiple range test.

## RESULTS

## Experiment 1

Both types of Gc produced E2 without the addition of substrates. Basal $E_2$ secretion was always significantly ($p < 0.05$) higher in MGc than in AGc (47.9 pg/$10^5$ cells vs 26.1 pg/$10^5$ cells, respectively; *Fig. 1*).

The addition of testosterone to the medium decreased $E_2$ secretion by MGc (47.9 pg/$10^5$ cells in control vs 34.2 pg/$10^5$ cells in testosterone-stimulated cultures; *Fig. 1*). $E_2$ secretion fold after the addition of testosterone to the medium of AGc (26.1 pg/$10^5$ cells in control vs 63.2 pg/$10^5$ cells in testosterone-stimulated cultures; *Fig. 1*).

Adding aromatase inhibitor to the medium of both types of Gc significantly decreased $E_2$ secretion (34.2 pg/$10^5$ cells in testosterone-stimulated MGc cultures vs 13.25 pg/$10^5$ cells, 14.5 pg/$10^5$ cells and 11.56 pg/$10^5$ cells after $1 \times 10^{-3}$ M, $0.5 \times 10^{-3}$ M and $0.1 \times 10^{-3}$ M aromatase inhibitor, respectively; 63.2 pg/$10^5$ cells in testosterone stimulated AGc cultures vs 18.97 pg/$10^5$ cells, 19.47 pg/$10^5$ cells, 22.05 pg/$10^5$ cells after $1 \times 10^{-3}$ M, $0.5 \times 10^{-3}$ M and $0.1 \times 10^{-3}$ M aromatase inhibitor, respectively; *Fig. 1*).

*Fig. 1.* Estradiol secretion by antral granulosa cells (AGc) and mural granulosa cells (MGc) under the influence of Testosterone alone and in combination with $1.0 \times 10^{-3}$ M (AR1), $0.5 \times 10^{-3}$ M (AR2) or $0.1 \times 10^{-3}$ M (AR3) aromatase inhibitor CGS 16949 A.
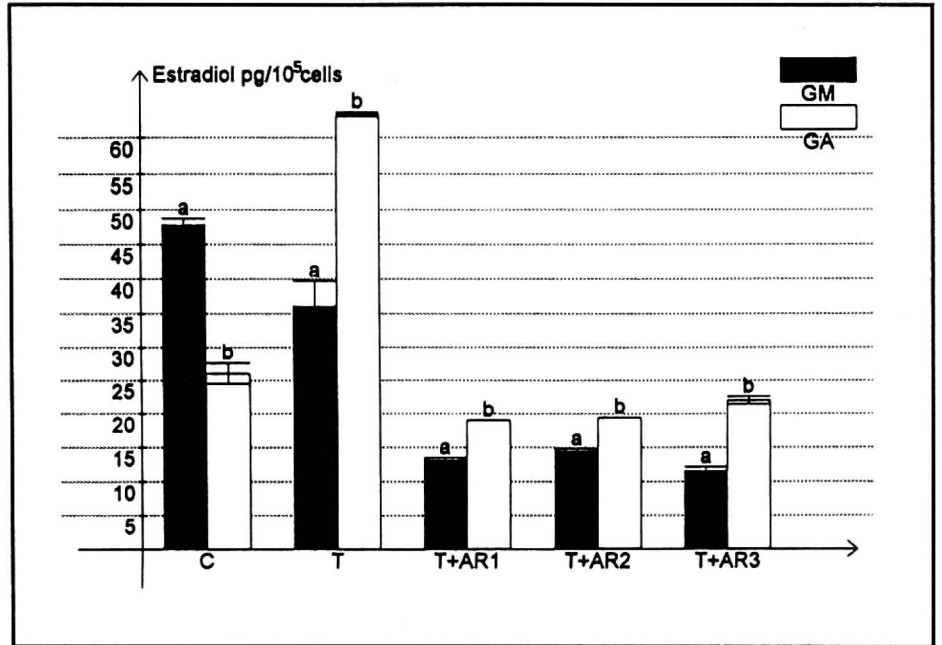
## Experiment 2

LH (100 ng) had no effect on $E_2$ production by both Gc types (*Fig. 2 a*). An additive influence of LH given together with testosterone was observed in MGc as well as AGc (39.45 pg/$10^5$ cells in LH-stimulated MGc vs 173.0 pg/$10^5$ cells
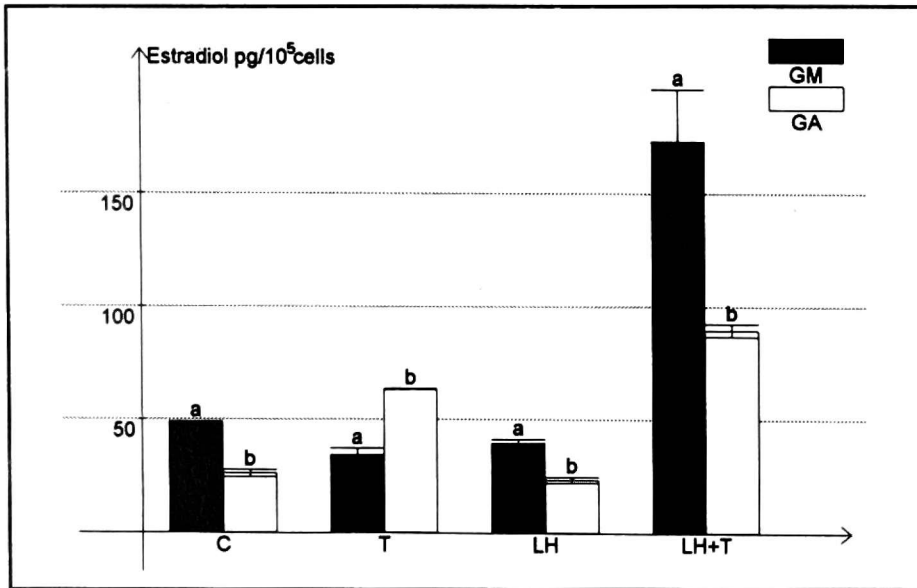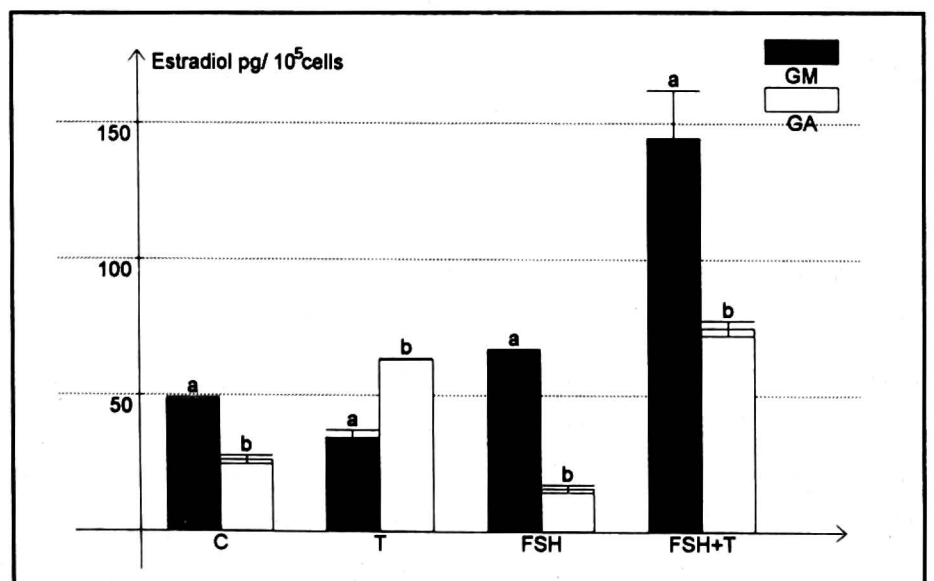


*Fig. 2 a.* Estradiol secretion by antral granulosa cells (AGc) and mural granulosa cells (MGc) under the influence of LH alone and in combination with Testosterone.



*Fig. 2 b.* Estradiol secretion by antral granulosa cells (AGc) and mural granulosa cells (MGc) under the influence of FSH alone and in combination with Testosterone.

in testosterone-treated cultures; 23.18 pg/$10^5$ cells in LH-stimulated AGc vs 90.55 pg/$10^5$ cells in testosterone-treated cultures; *Fig. 2a*). Thus, the stimulatory effect could be rather ascribed to testerone.

FSH not only significantly increased $E_2$ secretion (1.5 times) by MGc but also caused a marked increase of $E_2$ secretion (67.5 pg/$10^5$ cells in FSH-stimulated cultures vs 141.0 pg/$10^5$ cells in testosterone-treated cultures) when was added to the medium containing testosterone (*Fig. 2b*). However, FSH alone decreased $E_2$ secretion by AGc; an additive influence was observed when it was given together with testosterone (16.15 pg/$10^5$ cells in FSH cultured AGc vs 74.85 pg/$10^5$ cells in testosterone-treated cultures; *Fig. 2b*).

## DISCUSSION

In the present study, we observed that basal $E_2$ secretion by MGc was 2 fold higher than by AGc. The greater production of $E_2$ by MGc could have been caused by proximity to theca cells, which are a source of androgen substrate *in vivo*. This accords with Ford and Lunstra (11) who showed that after enzymatic disruption MGc had greater estradiol production than AGc when cultured initially, and they mainteined this advantage through day 8 when stimulated with FSH. However the basal $E_2$ secretion by AGc is lower than by MGc we observed that this cells have a greater ability to aromatize exogenous testosterone. Adding testosterone to the culture media coused a high increase of $E_2$ secretion by AGc. The slight decline of $E_2$ production observed in MGc after adding testosterone was probably the result of a suppresive action of high basal level of estradiol produced by MGc. The concentration of estrogen in cultured media of MGc was sufficiently high to cause negative feet back in $E_2$ production. Although $E_2$ production by AGc did not reach that of MGc, these cells retained some response to FSH. It is possible that during cell isolation cytokines secreted by white blood cells entangled to antral granulosa cells (12, 13). Synergism of FSH and testosterone in stimulation of $E_2$ production was observed in cultured Gc collected from both mural and antral granulosa cells. This is in agreement with Moon *et al.* (14) who concluded that FSH regulates $E_2$ biosynthesis in hypophysiectomised rat ovary by a specific stimulating action on the aromatizing enzyme system. The indirect studies suggested that the side-chain cleavage enzyme system was substrate limited and rised the possibility of cholesterol biosynthesis as a possible additional site for regulation (15). Nimrod *et al.* (16) suggested the main step in the biosynthesis pathway that is affected by FSH and androgen is the mitochondrial side-chain cleavage enzym system. Urban *et al.* (17) using primery serum-free cultures of immature porcine granulosa cells demonstrated that physiological concentrations of FSH *in vitro* stimulate the accumulation of

specific mRNA-encoding cytochrome P450scc in a time and dose-dependent manner. They suggested that FSH-stimulated P450scc mRNA accumulation itself results in part from cAMP-mediated increase in transcription of P450 scc gene. MGc are the most biochemically differentiated of the growing antral follicles, as indicated by their greater content of P450scc (18) and their hCG-binding capacity (19). The lack or slight decrease effect of FSH on $E_2$ production by AGe observed in our data could be explained by the low basal level of $E_2$ produced by this type of cells. This accords with Hiller and De Zwart (20) who omitted biologically active androgen from granulosa cell culture medium and obtained an aromatase response to hFSH at least 95% lower than that elicited by a combination of hFSH with testosterone. Barreca *et al.* (21) showed that IGF prevalently expressed by Gc is IGF-II, whose synthesis can be stimulated by FSH, while IGF-I seems to be prevalently expressed by theca cells (22, 23). It was also showed by Barreca *et al.* (24) that IGF-I significantly stimulated $E_2$ production by Gc in culture in a manner comparable to that of FSH. So, taken into consideration fact that MGc could be under the influence of the factors secreted by theca cells, IGF-I could be the additional agent responsible for high basal amount of $E_2$ secreted by MGc and high stimulatory effect of FSH added alone on $E_2$ secretion by this type of cells. This explanation requires additional confirmation. LH alone had no effect on $E_2$ production by both type of cells. It was also observed by Gregoraszczuk (25) in the culture of luteal cells collected from early and midluteal phase. Adding testosterone increased 4.3 fold and 3.8 fold $E_2$ secretion by MGc and AGc, respectively, over secretion observed after adding of LH alone. On the other hand a possitive synergism between LH and testosterone on $E_2$ secretion in both type of granulosa cells was observed.

In conclusion; this data provide the basis for future analysis of the potential differences in this two type of cells: first depends on the follicular phase and second depends on the influence of the agent secreted by theca cells.

## REFERENCES

1. Kasson B, Meidan R, Davoren B, Hsuett AJW. Identification of Subpopulations of Rat Granulosa Cells: Sedimentation Properties and Hormonal Responsiveness. *Endocrinology* 1985; 117: 1027—1034.
2. Rao IM, Mills TM, Anderson E, Mahesh VB. Heterogeneity in Granulosa Cells of Developing Rat Follicles. *The Anatomical Record* 1991; 229: 177—185.

3. Zoller LC, Weisz J. Identification of cytochrome $P_{450}$, and its distribution in the membrana granulosa of the preovulatory follicles, using quantitative cytochemistry. *Endocrinology* 1978; 103: 310—313.

4. Amsterdam A, Koch Y, Liberman ME, Lindner HR. Distribution of binding sites for human chorionic gonadotropin in the preovulatory follicle of the rat. *C Cell Biol* 1975; 67: 894—900.

5. Oxberry BA, Greenwald GS. An autoradiographic study of the binding of ^{125}I-labelled FSH, hCG, and PRL to the hamster ovary throughout the estrus cycle. *Biol Reprod* 1982; 27: 505—516.

6. Zoller LC, Weisz J. A quantitative cytochemical study of glucose 6-phosphate dehydrogenase and 3βHSD dehydrogenase activity in the membrana granulosa of the ovulable type of follicle in the rat. *Histochemistry* 1979; 62: 125—135.

7. Zeleznic AJ, Midgley AR, Reichart LE. Granulosa cell maturation in the rat: Increased binding of human chorionic gonadotropin following treatment with follicle stimulating hormone *in vivo*. *Endocrinology* 1974; 95: 818—825.

8. Chaning CP, Ledwitz-Rigby F. Physiology and genetics of reproduction, part B Plenum Publ Corp New York 1975.

9. Stoklosowa S. Tissue culture of gonadal cells. *Acta biol Acad Sci hung* 1982; 33: 367—379.

10. Stokłosowa S, Gregoraszczuk EL, Chaning CD. Estrogen and progesteron secretion by isolated cultured porcine thecal and granulosa cells. *Biol of Reprod* 1982; 26: 943—952.

11. Fird JJ, Lunstra DD. Differential Production of Estradiol by Subpopulations of Porcine Granulosa cells. *J Reprod Develop* 1992; 38: 91—98.

12. Droesch K, Fulgham DL, Liu H, Rrosewaks Z, Alexander NJ. Distribution of T cell subsets in follicular fluid. *Fert Steril* 1988; 50: 618—621.

13. Loukides JT, Loy RA, Edwards R, Honig J, Visintin I, Polan ML. Human fluids contain tissue macrophages. *J Clin Endocrinol Metab* 1990; 71: 1363—1367.

14. Moon JS, Duleba A, Leung PCS, Gomel V. Metabolism of testosterone by human granulosa cell in culture. Influence of follicle stimulating hormone and luteinizing hormone. *Am J Obst Gynec* 1982; 142: 692—697.

15. Lino J, Baranao S. Hammond JM. Multihormone regulation of steroidogenesis in cultured porcine granulosa cells: studies in serum-free medium. *Endocrinology* 1985; 116: 2143—2151.

16. Nimrod A. On the synergistic action of androgen and FSH on progestin secretion by cultured rat granulosa cells. Cellular and mitochondrial cholesterol metabolism. *Moll Cell Endocrinol* 1981; 21: 51—62.

17. Urban RJ, Garmey JC, Shupnik MA, Veldhuis JD. Follicle-Stimulating Hormone increases concentrations of Messenger Ribonucleic Acid encoding cytochrome P450 cholesterol side-chain cleavage enzym system in primary cultures of porcine granulosa cells. *Endocrinology* 1991; 128: 2000—2007.

18. Goldschmit D, Kraicer P, Orly J. Preovulatory expression of cholesterol side-chain cleavage cytochrome $P_{450}$ in cumulus cells. *Endocrinology* 1989; 124: 369—378.

19. Oxberry BA, Greenwald GS. An autoradiographic study of the binding of [125]J-labelled follicle-stimulating hormone, human chorionic gonadotropin and prolactin to the hamster ovary throughout the estrous cycle. *Biol Reprod* 1981; 27: 505—516.

20. Hiller SB, De Zwart FA. Evidence that granulosa cell aromatase induction activation by follicle-stimulating hormone is an androgen receptor-regulated process in vitro. *Endocrinology* 1981; 109: 1303—1305.

21. Barecca A, Artini PG, Arvigo M. Interrelationships between follicle stimulating hormone and the the growth hormone- insulin-like-growth hormone-insulin-like-growth factor-IGF-binding protein axes human granulosa cells in culture. *J Endocrinol Invest* 1996; 19: 35—42.

22. Hernandez ER, Huwitz A, Vera A, Pellicer A. Expression of the genes encoding the insulin-like growth factors and their receptors in human ovary. *J Clin Endocrinol Metab* 1992; 74: 19.

23. El-Roei A, Chen X. Expression of the genes encoding insulin-like growth factors the IGF and insulin receptors, and IGF-binding proteins-1-6 and the localization of their gene products in normal and polycystic ovary syndrome ovaries. *J Clin Endocrinol Metab* 19974; 78: 1488.

24. Barreca A, Artini PG, Del Monte P, Ponzani P. In vivo and in vitro effect of growth hormone on estradiol secretion by human granulosa cells. *J Clin Endocrinol Metab* 1993; 77: 61.

25. Gregoraszczuk EL. The Interaction of Testosterone and Gonadotropins in Stimulating Estradiol and Progesterone Secretion by Cultures of Corpus Luteum Cells Isolated from Pigs. *Endocrinol Japon* 1991; 38 (3): 229—237.

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