STRUCTURAL VARIATIONS PRODUCED IN VITRO BY GONADOTROPHINS AND STEROID HORMONES ON THE CELL SURFACES OF THE OVARIAN EPITHELIUM OF THE CHICK EMBRYO

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SUMMARY

The purpose of the present work was to analyze the structural variations produced "in vitro" by hormones on the cell surfaces of the ovarian epithelium of the chick embryo, in relation to the growth of the left ovary and atrophy of the right one. Explants of both ovaries from embryos at 7 to 19 days of development were separately cultured for 4 days in MEM with 10 % of fetal bovine serum (control), and plus 17-β-estradiol, testosterone propionate, progesterone, FSH, LH or hCG added individually to cultures (experimental). The cultures were processed for their structural, ultrastructural and cytochemical study. In control cultures, membrane differentiations and mucosubstances were similar in both ovaries and at all ages to those in ovo. 17-β-estradiol produced a greater development of microvilli, junctional complexes and mucine in the epithelial cells and interdigitations in the germ cells of the left ovary, while in the right gonad there was cell regression. Testosterone and progesterone evoked in the left gonad a response similar to that obtained with the estrogen, whereas in the right ovary no changes were observed with respect to controls. FSH led to cellular regression in both ovaries. Finally, with LH or hCG the changes produced in the left ovary were similar to those induced by the estrogen, and in the right one an increase of membrane differentiations and of mucosubstances related to them was found in comparison to controls. The results obtained in explants of ovaries at 7 days of development and cultured for 4 days, indicate that the modifications of the cell surfaces of the ovarian epithelium of the chick embryo would be related to the action of steroid and gonadotrophic hormones at the start of gonadal differentiation.
Key words: Ovaries - Chick embryo - Gonadotrophins - Steroids - Cultures.

INTRODUCTION

In previous studies from our laboratory, it was demonstrated that in vitro, gonadotrophic and steroid hormones participate in the development of the functional left ovary and in the atrophy of the right one (3, 4, 5, 7, 8).

It could be established that hCG and LH induce differentiation of the left ovary, producing a greater development of its cortex, and an increment of interstitial cells in the medulla. Estrogens and testosterone exert a similar action, provoking especially a pronounced cortical development in the left ovary. In the right ovary which does not develop a cortex during embryogenesis and undergoes regression, hCG and LH stimulate a cortical formation similar to the left funcional one. Estrogen accelerates the process of cellular involution and testosterone induces the incipient formation of on "ovarian" cortex.

It has also been shown "in ovo" that membrane-associated glycoproteins and

glycosamineglycans of epithelial and germ cells increase in the left ovary, while these mucosubstances diminish simultaneously with involution in the right gonad (1, 2, 24, 25). Cuming and Dubois (10) showed that the germinal epithelium of the left ovary exerts a chemotactic attraction on germ cells, due to production of glycoproteins by its own cells.

The importance of membrane differentiations and coats in migration, growth and celular differentiation has been demonstrated in diverse experimental models, not only in embryogenesis but also in cancer, metastases etc., Fabro and coworkers (12) found differences in the ultrastructural and biochemical characteristcs of membrane-specialized contacts in the ovarian epithelium of the chick embryo, dependent of the age and the ovary studied, and Letourneau and coworkers (20) established that estrogens act on junctional complexes and nexus in tumor cells but not in normal ones.

The purpose of this work was to anlyze the structural, ultrastructural and cytochemical variations produced in vitro by gonadotrophic and steroid hormones on membrane differentiations and mucosubstances of epithelial and germ cells of the ovarian epithelium.

MATERIAL AND METHODS:

Cobb's White Rock chick embryos at 7, 11, 15 and 19 days of incubation were used. Stages of development were determined according to the classification of Hamilton and Hamburger (15). The gonads, free of mesonephros, were cut in small pieces under aseptic conditions. Explants of both ovaries were separatedly cultured for 4 days in "Eagle's Minimum Essential Medium, with the addition of 10 % fetal bovine serum, 1 % L-glutamine, 100 U/ml penicilin and 5 mg/streptomycin sulphate, at 37°C and in an atmosphere of 5 % C02/95 % 0_2 (controls). To another group of cultures (experimental), FSH (30μg/ml), LH (30μg/ml), hCG (15UI/ml), 17-β-estradiol (1μg/ml) or progesterone (10μg/ml) were added singly to different explants. Hormones were supplied by United States Biochemical Corporation. Gonadotrophins were dissolved in 0.9 % Na Cl, while stock solutions of steroid hormones were prepared in absolute ethanol. At the highest concentration used in this study (1.4×10^{-2} mM) this solvent did not have any effect on the cultures (6).

At the end of each culture period the explants were processed for the different studies. For the structural and cytochemical analysis, the explants were fixed in 10 % neutral formaldehyde at pH 7.4 in phosphate buffer and in Bouin's fluid. Serial slices were obtained and stained with the following techniques:

1) Hematoxylin-eosine, 2) Toluidine Blue at pH 3.8 to determine alcohol-resistant basophilic and metachromatic substances, 3) PAS to detect the presence of glycoproteins. Control slices were exposed to enzymatic digestion with sialidase, 4) Periodic acid-methenamine- silver to study complex carbohydrates; 5) Alcian Blue at pH 2.5 and 1.0 for the analysis of sulphated and non sulphated acid glycosamineglycans. Control slices were exposed to blocking reactions (methylation) and saponification.

For the ultrastructural study slices were fixed in Karnovsky solution (18) for 2 h and then postfixed for 1 h in 1 % osmium tetroxide, dehydrated in acetone and embedded in Araldite. Thick slices were stained with Toluidine Blue. Fine slices were stained with uranyl acetate, contrasted with lead citrate and examined with a Siemens E 101 electron microscope.

Glycoconjugates were ultrastructurally detected by means of a policationic salt of ruthenium Red. Slices were fixed in a diluted Karnovsky solution containing 0.1 % ruthenium Red for 30 min. After washing they were postfixed in 1 % osmiun tetroxide containing 0.075% ruthenium Red for 60 min. They were finally processed with the conventional technique for electron microscopy (11) with or without lead citrate staining.

RESULTS

The most remarkable results were obtained in 4 days-cultures from ovaries at 7 days of development.

Controls: The left ovarian epithelium presented stratified epithelial cells surrounding gonocytes and a discontinous basement membrane. Superficial epithelial cells, connected by junctional complexes, contained scant microvilli in their apical surface. Germ cells were characterized by the presence of numerous and irregular digitations. Both cell types had an alcianophilic, PAS, methenamime-silver and ruthenium Red positive coat, that was metachromatic with Toluidine Blue.

The right ovary presented a simple columnar epithelium whose cells surrounded scarce germ cells. Epithelial cells possessed tight and intermediate unions as well as desmosomes; microvilli were found in their free surface. Gonocytes had regular contours without interdigitations. Cytochemical staining showed a weekly positive reaction of mucosubstances on the membrane coats.

Effect of FSH: Epithelial and germ cells of both ovaries presented vacuoles in the cytoplasm, multilamellar formations and mitochondrial tumefaction. These involutive signs were associated with a diminution of cytochemical staining with respect to controls.

Effect of LH and hCG: Both hormones acted similarly on both ovaries. Thus in the left ovary, the stratified epithelium presented a notorious development of union complexes in the superficial epithelial cells and in the free apical portion, as well as microvilli and pinocytotic vesicles. Tight unions maintained a constant presence and distribution, and were found forming a row that started at the apex of the cells. Multiple points of fusion of contiguous membranes appeared in this zone, separated by regions of unfused membranes. A fibrogranular condensation of the cytoplasm was notorious next to membranes. Continuing the tight union, the intermediate zone was found, in which the intercellular space was evident and the opposed membranes were reinforced by a cytoplasmic condensation reached by interlaced filaments. The characteristic desmosomes typical of this region stood out from the other contacts by the presence of a granular filamentous material between the cell membranes, that appeared thickenned by the subjacent cytoplasmic condensation, reached also by numerous filaments. Deep epithelial cells had numerous microvilli in all their contour while intercellular contacts were scarce.

The gonocytes located among epithelial cells presented numerous microvilli and prolongations of epithelial and germ cells, that were coated by strongly alcianophilic mucosubstances, that were PAS, methenamine silver and ruthenium Red positive, showing also alcohol-resistant metachromasia with Toluidine Blue.

The response of the right ovary to LH or hCG was similar to that of the left one and, at a difference with the control, the epithelium was stratified. Superficial epithelial cells had few microvilli and junctional complexes whereas the deepest cells showed numerous microvilli and scant development of intercellular junctions (Fig. 1 A). The PAS positive and periodate-reactive coats were evident on membrane microvilli and interdigitations of the different cell types. Digestion with amilase did not modify PAS positivity while sialidase had a slight effect. With ruthenium Red, a finely granular component was seen, coating the different cellular projections and their surfaces. This granular material formed an homogeneous film in some areas that had a heavier concentration of mucosubstances (Fig 1 B). Alcianophilic (at both pH 2.5 and 1.0) and metachromatic coats were also observed. Methylation and saponification reactions indicated the presence of sulphated and non sulphated acid glycosamineglycans, being the first ones predominant.

Effect of 17-β-estradiol: An increase of apical microvilli and pinocytotic vesicles was found in the superficial epithelial cells from left ovary explants. Typical junctions that were more developed than in controls were also seen connecting these cells. Deep epithelial cells showed abundant microvilli in all their contour and scarce junctions. Germ cells, interspersed among the epithelial

ones, possessed numerous interdigitations. Cytochemically, the presence of PAS, methenamine-silver and ruthenium Red positive mucosubstances was demostrated. These cells presented also an intense alcianophilia and metachromasia.

In the right ovary, 17-β-estradiol produced a regression of the epithelial and germ cells of the explants, although some desmosomes were maintained. The diminution of mucosubstances with respect to controls was remarkable.

Effect of testosterone and progesterone: The response of the epithelium of the left gonad was similar to that obtained with the estrogen, whereas no modifications appeared in the right gonad in comparison with controls.

DISCUSSION

The results obtained in 4 days-cultures of control 7 days-old ovaries demonstrate that the germinal epithelium behaves in vitro in a different manner in the functional left gonad and in the atrophic right one. In the left gonad we observed a stratified epithelium with superficial cells having scarce microvilli, mucosubstances and junctional complexes, in marked contrast to the deep layer, which presented epithelial cells with abundance of the above elements. These findings are in accordance with those of Grinnel (14); Hay (16) and Didier and coworkers (11), who proposed that mucosubstances would play an important role by way of their interaction with the surface of migrating cells. In this manner, epithelial and germ cells would migrate to form the cortex of the functional ovary.

The right ovary, which does not develop a cortex, showed a reduced amount of membrane specializations and mucosubstances of simple epithelial cells and few gonocytes.

These results indicate the absence of migration in this gonad; hence, the cortex would not be formed and the organ involutes.

The results obtained with the different hormones employed in this study on the germinative epithelium of the ovaries confirm and add further evidence on the importance of membrane specializations and mucosubstances for the processes of differentiation or atrophy in these organs (3, 6, 21). The cell involution induced by FSH in the left ovary has also been reported in vitro by Pittini and Milano (23).

Addition of LH induced the appearance of a stratified epithelium both in left and right ovarian explants, being remarkable the presence of junctional complexes in the superficial cells of the epithelium, contrasting with their absence in the deep epithelial layer. On the other hand, deep cells had numerous microvilli covered by mucosubstances in a greater proportion than in controls. Thus, it seems quite possible that the mucosubstances present in the surface of epithelial and germ cells and even in the extracellular matrix of both ovaries contribute to cortical formation (3, 24).

On the other hand, the junctions present in superficial cells would not allow their multiplication and migration. The presence of junctional complexes in tumor cells is considered of prognostic importance, insofar as their reduction and the appearance of microvilli with increment of mucosubstances would confer to tumor cells the potential to multiply and migrate (9, 17, 22). We have also observed the relationship between epithelial and germ cells, and think that the maturation and multiplication of gonocytes depends on epithelial cells (3, 6).

17-β-estradiol produced effects similar to those of LH and hCG on the left ovary, while the right ovary underwent regression. These result agree with those obtained by Gasc (13), who described a lack of receptors in the ovarian epithelium of the chick, and by Merck and coworkers (20) in granulosa cells of the rat ovary.

It may be concluded that the differentiation process of the functional left ovary would be related to the presence of membrane differentiations and mucosubstances in the ovarian epithelium, thus allowing a "nidation" of the germ cells. Epithelial and germ cells would migrate jointly to form the cortex.

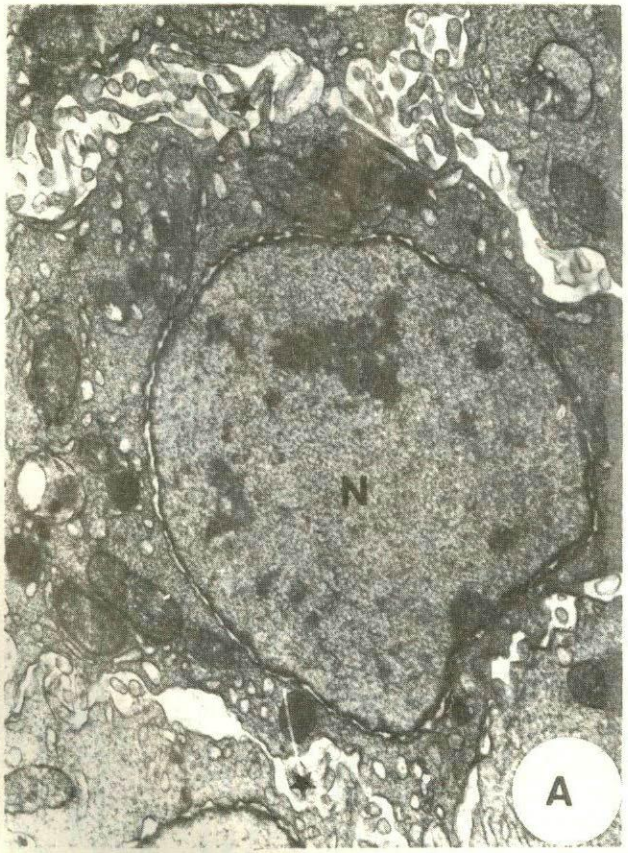
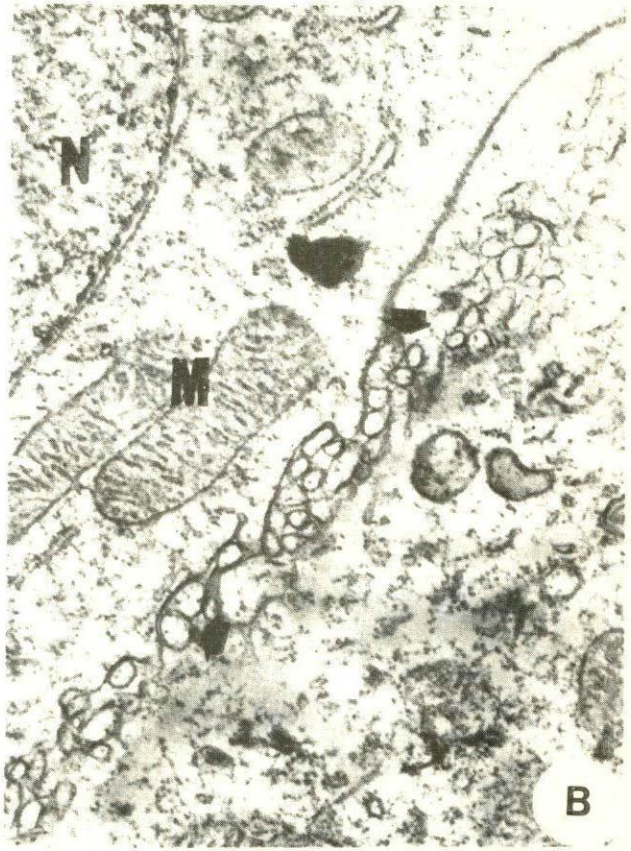


Figure 1: 7 days-old right ovary cultured for 4 days with hCG.
A: Deep epithelial cells with numerous microvilli (stars).Nuclei (N). 9,000X.



B: Ovocytes with Ruthenium Red positive interdigitations (arrows).Nuclei (N). Mitochondria (M). 18,000X.

The strikingly different morphology and cytochemistry of the right ovary, in which cortical progression is lacking, suggests a failure in some step (5) of the "mechanism that could impede the atrophy of this organ.

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RESUMEN

El objetivo del presente trabajo fue analizar las variaciones estructurales producidas in vitro por hormonas en las superficies celulares del epitelio ovárico del embrión de pollo en relación con la maduración del ovario izquierdo y la atrofia del derecho. Explantos de ambos ovarios de embriones de 7 a 19 días de desarrollo in ovo fueron cultivados por separado durante 4 días en MEM con 10 % de suero fetal bovino (control), y más 17-β-estradiol, propionato de testosterona, progesterona, FSH, LH o hCG individualmente (experimental). Los cultivos fueron procesados para su estudio estructural, ultraestructural y citoquímico (PAS, Alcian blue, Azul de toluidina, metenamina-plata, Rojo de rutenio). En los controles de todas las edades en ambos ovarios, las diferenciaciones de membranas y mucosustancias fueron similares a lo descripto in ovo. Con 17-β-estradiol, en el ovario izquierdo se produjo un mayor desarrollo de microvellosidades, complejos de unión y mucinas en las células epiteliales y de interdigitaciones en las células germinales, en tanto que en el ovario derecho hubo regresión celular. Con testosterona y progesterona la respuesta en la gónada izquierda fue similar a la obtenida con estrógeno mientras que en el ovario derecho no hubo modificaciones con respecto al control. FSH produjo regresión celular en ambos ovarios. Finalmente, con LH o hCG los cambios fueron semejantes a los obtenidos con estrógeno en el ovario izquierdo, y en el derecho se observó un incremento de las diferenciaciones de membranas y mucosustancias relacionadas con las mismas, con respecto al control. Los resultados obtenidos en los explantos de 7 días de desarrollo in ovo, cultivados durante 4 días, nos indican que las modificaciones de las superficies celulares del epitelio ovárico del embrión de pollo estarían ligadas a la acción de las hormonas esteroideas y gonadotróficas en el momento de la diferenciación gonadal.

Palabras claves: Ovarios - Embrión de Pollo - Gonadotrofinas - Esteroides - Cultivos.