Structure and Steroidogenic Activity of the Granulosa Layer of F1 Preovulatory Ovarian Follicles of the Hen (*Gallus domesticus*)

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The study was performed to determine the structure and steroidogenic activity of granulosa cells derived from the germinal disc region, proximal region and distal region of the largest preovulatory ovarian follicle (F1) of the hen. The study was carried out on 34 Hy-Line Brown egg-laying hens aged 40 weeks. Morphology of the granulosa cells was studied by histological assessment and scanning electron microscopy. Moreover, the level of P_4, histochemical activity of 3β-HSD and expression of 3β-HSD gene mRNA in granulosa cells of F1 follicle were determined. The findings indicate that the morphology and steroidogenic activity of the granulosa layer in F1 preovulatory ovarian follicle are associated with the region of the follicle. This is consistent with earlier studies. In the germinal disc region the granulosa cells form a multilayer while in the proximal and distal regions granulosa cells form a single layer. Analysis of P_4 concentration revealed that its level in granulosa cells was markedly reduced closer to the germinal disc. Moreover, our study demonstrates for the first time the lower histochemical activity of 3β-HSD and expression of 3β-HSD mRNA in granulosa cells from the germinal disc region compared with the proximal and distal region.

Key words: Hen, F1 preovulatory ovarian follicles, granulosa layer.

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The follicular wall of ovarian follicles in birds is a complex structure that includes follicular cells and the surrounding theca (theca folliculi) built of connective tissue. These cells are responsible for transport of nutrients and mechanical support of growing oocytes. They also play very important roles for the development of the oocyte and ovulation. One of the major functions of this tissue is biosynthesis of steroids (BAHR *et al.* 1883; BAKST *et al.* 1983; MARRONE & SEBRING 1989; JOHNSON 1990; NITTA *et al.* 1993). The ovary of a mature hen generally contains 5-6 large yellow preovulatory follicles arranged in a follicular hierarchy, several postovulatory follicles, and numerous small follicles which have not entered the follicular hierarchy and are classified according to size: stromal follicles (<1mm) embedded in the ovarian stroma, small white follicles (1-4 mm) and large white follicles (4-8 mm). Follicles in the hierarchy are in a rapid growth phase and are classified according to size with the largest (F1) follicle destined to ovulate next, the second largest (F2) follicle to ovulate the following day, and so forth.

The structure and function of granulosa cells from preovulatory ovarian follicles, which are the principal source of progesterone (P_4) in avian ovaries, and their role in ovarian follicle growth has been the subject of several studies (ARMSTRONG 1979; BAHR *et al.* 1983; GOMEZ *et al.* 1998; NITTA *et al.* 1993; YOSHIMURA *et al.* 1993; YAO & BAHR 2001a; PROSZKOWIEC-WĘGLARZ *et al.* 2005; RZĄSA *et al.* 2009; SECHMAN *et al.* 2006; SECHMAN *et al.* 2009). Most of these studies regarded the granulosa layer as a uniform structure. Recent studies provide increasingly strong evidence that like in mammals, in which the granulosa layer is formed by two cell subpopulations, the morphology and function of preovulatory granulosa cells is not uniform and depends on where it is located relative to the germinal disc (YAO & BAHR 2001a, b).

Therefore, the objective of the present study was to demonstrate the structure of the granulosa layer

and to determine steroidogenic activity of granulosa cells derived from the germinal disc region, proximal region and distal region of F1 preovulatory ovarian follicles of the hen.

Material and Methods

The experiment was carried out on 34 Hy-Line Brown egg-laying hens at the age of 40 weeks and weighing an average of 2 kg. Birds were fed *ad libitum* and kept in individual batteries of cages under a photoperiodic regime of 16L–8D. Egg laying was monitored using a computerized recording system. Animals were decapitated one hour before ovulation to collect the largest F1 preovulatory ovarian follicles, from which the germinal disc region, proximal region and distal region were isolated.

The material for histological and ultrastructural analysis (obtained from 8 hens) was fixed in 2.5% glutaraldehyde and 1% osmic acid. After dehydration in increasing ethanol concentrations, histological material for light microscopy was embedded in Epon 812 and then sectioned with glass knives using a Tesla 490 A ultramicrotome. Sections 1 μm thick were stained with methylene blue and alkaline fuxin (Humphrey & Pittman 1979). Meanwhile, fixed material for ultrastructural analysis was coated with gold on a sputter coater (Jeol JFC 1100E) and analysed under a scanning electron microscope (JSM-5410).

The activity and location of 3β-hydroxysteroid dehydrogenase enzyme (3β-HSD) was detected using the histochemical method of Levy *et al.* (1959). F1 ovarian follicles (obtained from 10 hens) were frozen in liquid nitrogen, cut on a cryostat (Slee MEV, Germany) into 10 μm sections at -25°C, and incubated in incubation medium at 37°C, where pregnenolone (P$_5$) was used as a substrate. Enzyme activity sites were marked by dark blue formazan granules formed from NBT reduction. The intensity of histochemical reaction in granulosa cells was determined based on optical density measurements (gray scale with pixel values of 0 for white and 128 for black) using MultiScan v.14.02 image analysis system. The preparations were analysed using a NIKON E600 light microscope.

The expression of 3β-HSD gene mRNA (N=6) was quantified by real-time PCR. Granulosa layers of F1 follicles were manually separated by cutting the follicle with a razor blade and peeling the granulosa layer, first from the yolk and then from the theca layer (Gilbert *et al.* 1977). They were then washed with avian Ringer's buffer three times. Additionally, samples of the liver were isolated as a negative control. All tissues were placed into RNA later (Sigma, USA) and thereafter stored at -80°C until determination of gene expression. Total RNA was extracted with TRI Reagent® (Molecular Research Center, Inc., USA) according to the included protocol. The quantity and quality of the total RNA was ascertained by measuring absorbance at 260 and 280 nm with a spectrophotometer (Eppendorf, Germany). First-strand cDNA was synthesized by reverse-transcription of 1 μg total RNA with random primers and High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to the protocol provided by the manufacturer. The first strand cDNA was used for quantitative real-time PCR amplification with TaqMan MGB chemistry. Multiplex real-time PCR was performed in a 96-well thermocycler (StepOne Plus, Applied Biosystems, USA) according to the recommended cycling program (2 min 50°C, 15 s 95°C, 40 cycles of 15 s at 95°C, and 1 min at 60°C). As a reference gene 18S rRNA was used (Eukaryotic 18S rRNA Endogenous Control, Applied Biosystems; Gene Bank X03205.1; size of amplicon 187 bp). Assay-on-Demand, TaqMan MGB Gene Expression Kit (ID Gg03372858_s1) for 3β-HSD dehydrogenase (Gene Bank NM_205118.1; size of amplicon 125 bp) with TaqMan MGB probe (5'-FAM-CAACCAACCGCCACCTGGTCACTCT-NFQ-3') was used for analysis of enzyme mRNA expression in the granulosa cells of F1 follicles. All reactions were performed in five replicates and non-template controls were included. Expression level of 3β-HSD dehydrogenase was normalized with the 18 s RNA reference gene. The data were calculated according to the method of Livak and Schmittgen (2001) using the expression in GD granulosa cells as the calibrator (RQ = 1) and presented as RQ ± SD.

The concentration of P$_4$ in homogenised granulosa tissue from the three regions of F1 preovulatory ovarian follicles (N=10) was determined radioimmunologically (RIA). P$_4$ was determined according to Abraham *et al.* (1971) using [1,2,6,7,16,17-^{3}H] progesterone (spec. act. 96 Ci/mmol: Amersham International plc) as a tracer and an antibody (a gift from B. Cook, University of Glasgow, UK) against 11α-hydroxyprogesterone succinyl: BSA induced in a sheep. Cross-reactivity of the antibody was 1.9% with pregnenolone, 1.5% with corticosterone and less than 1% with other steroids. The sensitivity of the assay was 20 pg. Coefficients of variation within and between assays were below 5.0% and 9.8%, respectively. All

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samples were assayed in duplicate. The protein concentration was estimated using the method of LOWRY et al. (1951). The concentration of P_4 was computed in ng/mg protein and expressed as means ± SD.

The calculations were made using a one-way analysis of variance. Significant differences between the means were determined using Tukey's test at P≤0.05 level of significance.

Results and Discussion

Examination of the granulosa layer from the largest preovulatory ovarian follicles (F1) of the hen showed that it is structurally and functionally not uniform. In the germinal disc region, granulosa cells are arranged in several layers on a highly folded basement membrane (Fig. 1a). Cells resting directly on the basement membrane are elongated, and those located at higher levels, closer to the perivitelline membrane, are cube shaped. Further

away from the germinal disc, in the proximal and distal regions, cuboid granulosa cells form a single layer of cells (Fig. 1b, c). The observed heterogeneity of granulosa cells is related to their location in the preovulatory ovarian follicle. The distance to the germinal disc is assumed to play a key role in the nature and function of the granulosa layer. YAO and BAHR (2001a) demonstrated that the germinal disc plays a very significant role in granulosa cell differentiation and function. The germinal disc contains the nucleus and around 99% of the cell organelles of the oocyte. It is the growth centre of the developing follicle and a source of essential signals that keep the follicle alive. The germinal disc is also a rich source of autocrine and paracrine signals, which stimulate proliferation in the adjacent granulosa layer (VOLENTINE et al. 1998; YAO & BAHR 2001a, b). The fact that this region has strong proliferative properties is supported by TISCHKAU and BAHR (1996), who showed that the granulosa cells of the germinal disc region are directly stimulated by other growth factors, as a result of which they are less mature than are cells
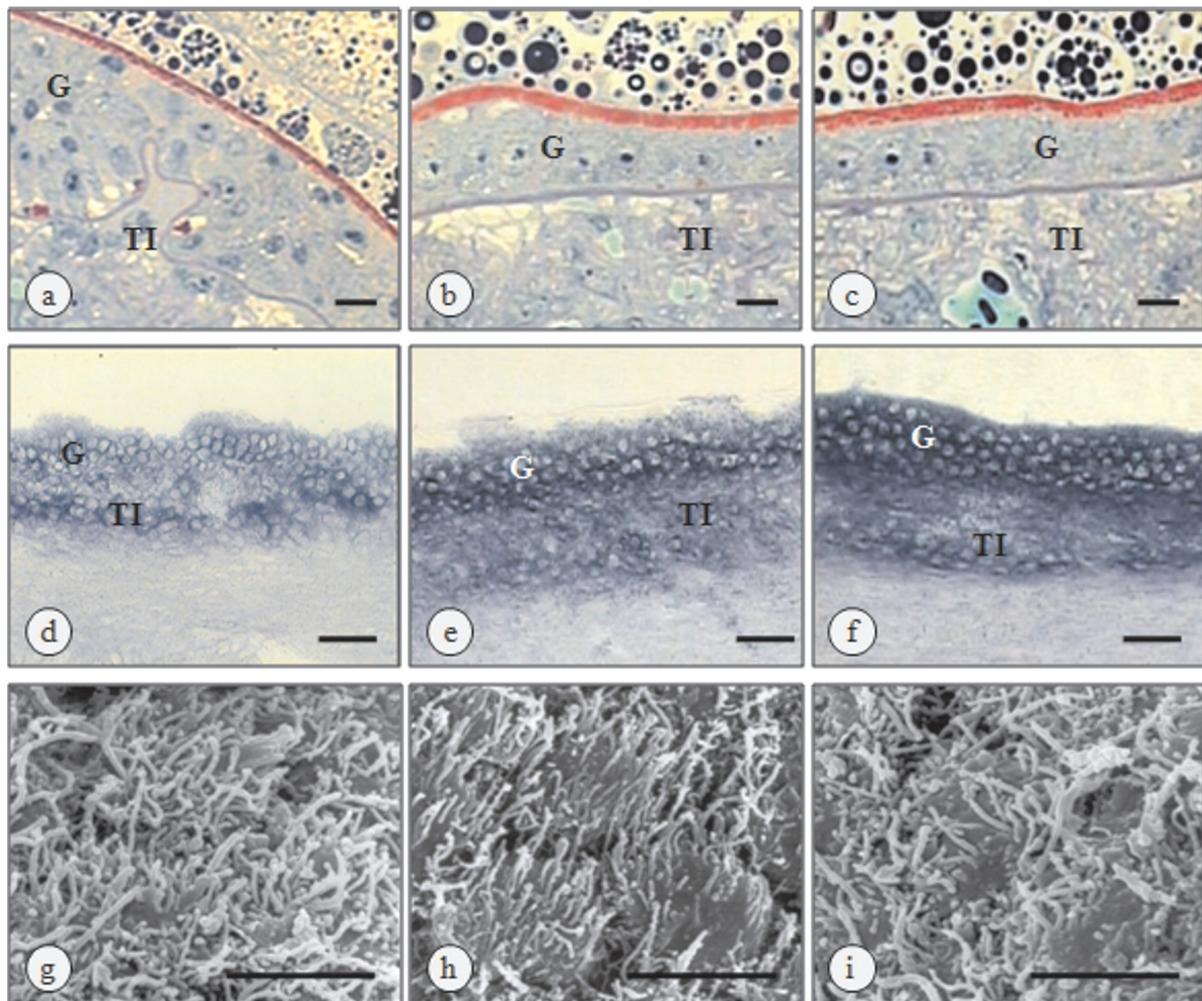

Fig. 1. Histological cross-section (a, b, c), Bar = 5 μm; histochemical localization of 3β-HSD (d, e, f), Bar = 20 μm; electronogram SEM (g, h, i), Bar = 5 μm of granulosa cells of the largest preovulatory ovarian follicles F1 of the hen – germinal disc region (a, d, g); proximal region (b, e, h), distal region (c, f, i): G – granulosa layer; TI – theca interna.
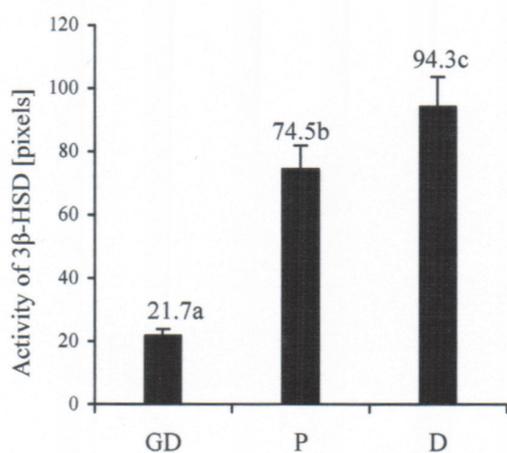
Fig. 2. Activity of 3β-HSD dehydrogenase in granulosa cells from the germinal disc region (GD), proximal region (P) and distal region (D) of the largest preovulatory ovarian follicles (F1) of the hen: a, b, c – significant at $P \leq 0.05$.

Fig. 3. Concentration of progesterone (P_4) in granulosa cells from the germinal disc region (GD), proximal region (P) and distal region (D) of the largest preovulatory ovarian follicles (F1) of the hen: a, b, c – significant at $P \leq 0.05$.
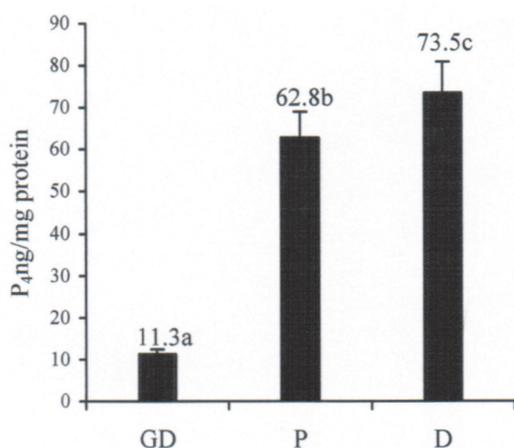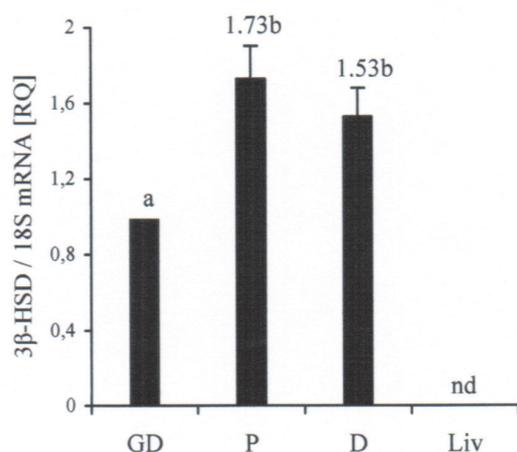

Fig. 4. Expression of 3β-HSD gene mRNA in granulosa cells collected from germinal disc region (GD), proximal (P) and distal (D) region of granulosa layer of F1 follicles. Data represent the mean of relative quantity (RQ) ± SD from five replicate experiments standardized to GD (RQ=1). The liver (Liv) was used as a negative control; nd – not detected: a, b – significant at $P \leq 0.05$.

located further away from the germinal disc. Likewise, MARRONE *et al.* (1990) showed that granulosa cells of the germinal disc region have a high mitotic index. TILLY *et al.* (1992) provided evidence that the granulosa layer derived from the germinal disc region of F1 preovulatory ovarian follicles possess a 2-fold greater level of ^{3}H-thymidine incorporation and 3-fold higher levels of plasminogen activator activity than the remaining granulosa layer. WANG *et al.* (1993) reported that plasminogen activator contributes to tissue remodeling during cellular growth. Meanwhile, the occurrence of a well-ordered single-layered structure of granulosa cells from the proximal and distal regions concurs with that reported by BAKST *et al.* (1979), who also found that granulosa cells from the distal region contain many lipid droplets, whose presence may be indicative of active steroid synthesis in this area of the granulosa layer.

The ultrastructural analysis performed in the present study using scanning electron microscopy showed that the surface of granulosa cells was covered by numerous processes (Fig. 1g, h, i). Similar structures (projections) were previously described in hens (WYBURN *et al.* 1965; PERRY *et al.* 1978; YOSHIMURA *et al.* 1993; YOSHIMURA & BAHR 1995) and geese (KOVACS *et al.* 1992). The authors of these studies suggested that the projections penetrate through the perivitelline envelope and come into direct contact with the oocyte, as a result of which they not only help substances pass from granulosa cells to the oocyte but also connect the oocyte with these cells. YOSHIMURA *et al.* (1993) reported in Japanese quail that the granulosa layer of the germinal disc region contains many cytoplasmic gap junctions with perivitelline membranes that allow communication between these cells and the oocyte. The germinal disc region is essential for the normal growth and development of the ovarian follicle. This is supported by YOSHIMURA *et al.* (1994), who showed in hens that destruction of the germinal disc region by freezing induces irreversible changes leading to arrested development of the follicle, anovulation and atresia of the preovulatory follicle. This suggests that for the ovarian follicle of the hen, the germinal disc and the adjoining granulosa layer are a key growth centre that controls follicular development, although the mechanism of action has not been adequately studied.

The enzymatic activity and expression of 3β-HSD mRNA of granulosa cells that we found in all F1 follicle regions of the analysed hens as well as the presence of P_4 in granulosa cells show that granulosa cells are involved in steroidogenesis, as confirmed by studies with hen ovarian follicles, which demonstrated that the granulosa layer of preovulatory follicles produces both P_4 and DHEA

(BAHR *et al* 1983; ROBINSON & ETCHES 1986; GOMEZ *et al.* 1998; LEE *et al.* 1998; PROSZKO-WIEC-WĘGLARZ *et al.* 2005; RZĄSA *et al.* 2009; SECHMAN *et al.* 2006; SECHMAN *et al.* 2009). Histochemical and immunohistochemical studies have also confirmed that granulosa cells of preovulatory follicles show 3β-HSD activity, which means that they are a site of steroid biosynthesis (ARMSTRONG 1979; DAVIDSON *et al.* 1979; MARRONE & SEBRING 1989; NITTA *et al.* 1993). However, with respect to this, our histochemical analysis also confirmed that granulosa cells differ in steroidogenic activity depending on their location (Fig. 1d, e, f). Accordingly, the activity of 3β-HSD dehydrogenase, a key enzyme for the Δ^4 pathway of steroid hormone synthesis, was the highest in the distal region, but it significantly decreased with decreasing distance to the germinal disc region (Fig. 2). Similar findings were noted for the P_4 hormone, the level of which decreased significantly closer to the germinal disc region (Fig. 3). This is also in agreement with the earlier study of TISCHKAU *et al.* (1997), who found the P_4 level (indicative of cell maturity) to be lowest in the granulosa layer of the germinal disc region and highest in the distal region. For this reason, the authors considered the contribution of the granulosa layer of the germinal disc region to overall progesterone production by the preovulatory follicle to be negligible. With respect to the expression of 3β-HSD gene mRNA in granulosa cells, the lowest level of 3β-HSD mRNA was found in the germinal disc region compared with the proximal and distal regions (Fig. 4). However, in contrast to our histochemical study, the abundance of 3β-HSD mRNA was not different in the proximal and the distal regions. The differences that we found between histochemical activity and expression of 3β-HSD mRNA in the proximal and distal region are unclear and further investigations are needed.

In conclusion, the morphological and functional differences that we found in the granulosa layer of F1 preovulatory follicles of the hen differ according to the region of the follicle. In the germinal disc region, the granulosa is multi layered, while in other areas of the follicle granulosa cells are arranged in a single layer. It was also shown that the steroidogenic activity of granulosa cells increases in the proximal and distal region compared with the germinal disc.

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