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Granulosa-Cumulus-Corona Expansion and Aromatase Localization in Preovulatory Follicles in Superovulated Heifers

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[1]Research Institute of Animal Production, Nitra, and [2]Department of Pathology, Veterinary University, Kosice, Slovak Republic, [3]Department of Anatomy and Physiology, The Royal Veterinary and Agricultural University, Frederiksberg, Denmark, [4]Endocrine Biochemistry Department, Medical Foundation of Buffalo, Buffalo, NY, USA, [5]Institut für Tierzucht und Tierverhalten (FAL) Mariensee, Neustadt, Germany, and [6]Veterinary University-Vienna, Vienna, Austria.

Laurincik, J, L. Kolodzieyski, P. Hyttel, Y. Osawa, H. Niemann, F. Schmoll, G. Brem, and L. Schellander: Granulosa-cumulus-corona expansion and aromatase localization in preovulatory follicles in superovulated heifers. Acta vet. scand. 1996, 37, 99-107. – Granulosa-cumulus and cumulus-corona expansion as well as aromatase localization within ovarian follicles were monitored during the preovulatory period in superovulated cattle that were blood sampled every 2'nd h for LH analyses. Granulosa-cumulus as well as cumulus-corona expansion were studied by means of transmission electron microscopy and computerized image analysis. Localization of aromatase, an enzyme involved in estrogen synthesis, was determined immunocytochemically using anti-human placental aromatase cytochrome P-450 antisera. Nuclear oocyte maturation was determined by aceto·orcein staining. Significant cell dissociation within the granulosa-cumulus stalk occurred before the breakdown of the germinal vesicle, i.e. the oocyte nucleus, during the period up to 5-7 h after the LH peak, i.e. the highest LH concentration during the surge. Significant increase in intercellular spacing between the cumulus-corona cells occurred at 13-15 and 19-21 h after the LH peak Before the LH peak all layers of granulosa cells were immunocytochemically stained for aromatase At 5-7 h after the LH peak, however, only the granulosa cell layers located near the basal lamina were stained, and at all later intervals staining was absent. The granulosa cells of primary and secondary follicles, the interstitial gland cells, the theca interna cells and the oocytes in all follicles were immunocytochemically unstained

cattle; oocyte; maturation.

Introduction

The preovulatory LH surge initiates cumulus oophorus expansion, resumption of meiosis and specific alterations of steroidogenesis within the preovulatory bovine follicle *(Dieleman et al.* 1983, *Callesen et al.* 1986, *Hyttel et al.* 1986a). About 9-12 h after the LH peak, i.e. the highest LH concentration during the surge, the gap junctional contact between the innermost cumulus cells, i.e. the corona cells, and the oocyte is broken *(Hyttel et al.* 1986a) and the steroidogenesis of the preovulatory follicles is redirected from being estradiol-17β (E2) dominated to become progesterone (P4) dominated *(Dieleman et al.* 1983, *Callesen et al.* 1986). The final step of the biosynthesis of

ovarian estrogen is known to be aromatization of androgen. Previous immunocytochemical studies on the localization of aromatase, an enzyme converting testosterone to E2 have documented that only the granulosa cells of the bovine antral follicles are residence for this enzyme *(Zhong et al.* 1989, *Laurincik et al.* 1994). However, the possible changes in the aromatase activity in relation to the occurrence of the LH peak have not been addressed.

It was the aim of the present study to monitor the granulosa-cumulus-corona expansion and aromatase localization within the bovine preovulatory follicle and relate these parameters to the occurrence of the preovulatory LH peak.

Materials and methods

Animal treatment and follicle recovery

Cyclic heifers (n = 33, Slovak pinzgau, 16-17 months old) were heat synchronized with 0.75 mg cloprostenol (Oestrophan, Spofa, Praha, CSFR). Animals, which exhibited a discernable corpus luteum on day 10 (n=30) were treated with a total dose of 20 mg (NIH) FSH-P (Folicotropin, Spofa, Praha, CSFR) given every 12 h in decreasing doses: 1×6, 1×4, 4×2, 2×1 mg, respectively, until they were slaughtered. Luteolysis was induced by administration of 0.75 mg cloprostenol 48 h after the initial FSH-P injection. Blood samples were obtained from the jugular vein using an indwelling cannula inserted 2 days before the first sampling. Sampling was initiated 18 h after the second injection of cloprostenol and was performed at 2 h intervals. Blood samples were collected into heparinized test tubes and were centrifuged immediately for 15 min at 2740 g. Plasma was stored at –20°C until LH analyses were performed. The animals were slaughtered at observation of maximal heat. Based upon results of the LH analyses the animals were retrospectively assigned to one of the following groups according to the occurrence of the LH peak: Group 1 (N = 4) was slaughtered before the occurrence of the LH peak, group 2 (N = 4) at 5-7 h, group 3 (N = 4) at 9-12 h, group 4 (N = 4) at 13-15 h, and group 5 (N = 4) at 19-21 h after the LH peak, respectively. Animals (N = 10) that did exhibit an LH surge were deleted from the material. Follicles >8 mm in diameter, which according to *Laurincik et al.* (1993) are considered to be preovulatory, were in each animal at each time interval assigned for observation of granulosa-cumulus-corana expansion, cumulus-oocyte disconnection, aromatase localization within the follicular wall, and nuclear oocyte maturation. Data from all follicles within each group were pooled.

Granulosa-cumulus and cumulus-corona expansion

Preovulatory follicles (>8 mm, *Laurincik et al.* 1993, N = 40, i.e. 2 per animal), were prepared for computerized image analysis *(Murdoch* 1988) according to the following modified protocol. The follicles were dissected from the ovary and fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH = 7.2) and cut into quadrants. The quadrant of the follicle that contained the oocyte was identified under a dissecting microscope. The granulosa-cumulus stalk and cumulus-corona-oocyte complex (either maintained as a single specimen, which was typically the case in group 1, or separated into 2, which was typical in groups 2-5) of each follicle was dissected free and post-fixed in 1% OsO_4 in 0.1 M cacodylate buffer, dehydrated and embedded in Epon following standard procedures *(Hyttel & Madsen* 1987). Subsequently, the specimens were sectioned into semi- and ultrathin sections. In specimens including stratum granulosum and cumulus oophorus the ultrathin sections were produced through the stalk and the equatorial plane of the oocyte. In free cumulus-corona-oocyte com-

plexes the ultrathin sections were prepared at the equatorial plane of the oocyte. Computerized image analysis on the intercellular spaces was performed on 10 adjacent ultrathin sections in each specimen. For the analysis of granulosa-cumulus expansion 10 random areas including the stalk of about 65×110 μm were photographed. For the analysis of cumulus-corona-oocyte complexes 10 random areas of a similar size were photographed at a distance of about 50 μm from the zona pellucida. Subsequently, prints of a final magnification of 2675× were produced, and image analysis was performed on an EDS X-ray Spectrophotometer Kevex Delta V (Kevex, Nex York, USA) following videodigitation of each print. The results of the analysis was expressed as the area occupied by intercellular spaces in percentage of the total analyzed area. Data for individual animals were subjected to a one-way analysis of variance. Specific means were compared with Duncan's New Multiple Range Test. Percentage data were transformed (arc sin%) for the purpose of statistical analyses *(Murdoch* 1988).

Aromatase localization

Preovulatory follicles (> 8 mm, *Laurincik et al.* 1993, N = 80, i.e. 4 per animal/16 per LH interval) were removed from the ovary by dissection, and the oocyte was localized under the stereomicroscope (125×). Pieces of follicular wall with the cumulus-corona-oocyte complex were frozen in liquid nitrogen as was the rest of ovarian cortex containing preantral and small antral follicles. Cryosections (6 μm) were cut at –25°C and fixed in a paraformaldehyde atmosphere for 10 min. After fixation, the specimens were rinsed in phosphate buffered saline (PBS) and incubated with rabbit anti-aromatase cytochrome P-450 antiserum (1:500) for 72 h at 4°C *(Zhong et al* 1989, *Ishimura et al.* 1989, *Yoshinaga-Hirabayashi et al.* 1990, *Laurincik et al.* 1994). After washing in PBS, the sections were

Table 1. Presence of preovulatory follicles in the ovaries of superovulated heifers relative to the time of the LH peak.

Group	Animals (N)	Follicles >8 mm (N)	Follicles per heifer (mean ± SD)
1	4	41	10 3 ± 0.7
2	4	42	10.5 ± 0.4
3	4	44	11 0 ± 0 4
4	4	46	11.0 ± 1 1
5	4	40	10.2 ± 0 9

Group 1 before LH peak; Group 2· 5-7 h after LH peak; Group 3: 9-12 h after LH peak, Group 4. 13-15 h after LH peak, Group 5 19-21 h after LH peak.

treated with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (Sevac, Praha, CSFR) for 2 h at room temperature and examined with a Nikon fluorescence microscope.

All the procedures for the purification of antigen (aromatase), the production of the antibody, and the specificity of the antibody have been previously described *(Osawa et al.* 1987 a,b). Control sections were treated with normal rabbit serum instead of the antiserum.

Nuclear oocyte maturation

The remaining preovulatory follicles (> 8 mm; *Laurincik et al* 1993) present in the ovaries in each animal (Table 2) were punctured and cumulus-corona-oocyte complexes attempted aspirated and isolated. The nuclear oocyte maturation was examined in whole mount preparations by phase-contrast microscopy after fixation in acetic acid: methanol (1:3 , V:V) and staining with 1% orcein *(Xu et al.* 1986). Nuclear oocyte maturation was also assessed in semithin sections of the cumulus-corona-oocyte complexes used for computerized image analysis.

LH analysis

LH concentration of peripheral blood was

Table 2. Nuclear maturation of oocytes relative to the time of the LH peak.

Time relative to the LH peak	Oocytes Examined N/mean[1] ± SD	Nuclear stages of oocytes GV %	GVBD %	MI %	MII %
Before	17/4.0 ± 0.2	100			
5-7 h after	18/4.5 ± 0.8	67	28	5	
9-12 h after	20/5.0 ± 0 2	5	45	50	
13-15 h after	19/4.7 ± 0.6		11	84	5
19-21 h after	18/4.5 ± 0.4			22	78

[1]Mean number of oocytes per heifer GV: Intact germinal vesicle, GVBD: germinal vesicle breakdown; MI: metaphase I, MII: metaphase II.

measured using a double antibody assay *(Stupnicki & Madej* 1976). Immunogene NIH-LH-B 9 was used to prepare the antibodies. The sensitivity of the assay was 50 pg bLH. Intra- and interassay coefficients of variation were 8.6% and 12.4%, respectively. The LH peak was defined as the highest LH concentration measured during the surge.

Results

Superovulatory response

The superovulatory response in each of the experimental groups in terms of preovulatory follicles is indicated in Table 1. As it appears from the table, the response was almost identical in the different groups.

Granulosa-cumulus and cumulus-corona expansion

The granulosa-cumulus expansion of preovulatory follicles as analyzed by computerized image analysis is presented in Fig. 1. The intercellular spaces in the stalk (granulosa-cumulus) increased significantly 5-7 h after the LH peak. Only a minor increase in intercellular spacing between the cumulus-corona cells was noted during the first 2 intervals after LH peak. However, an increase occurred at 13-15 and especially 19-21 h.

Aromatase localization

Before the LH peak, all granulosa cell layers of the preovulatory follicles displayed immunocytochemical staining demonstrating aromatase localization (Fig. 2A). The cumulus and corona cells as well as the oocytes lacked staining. At 5-7 h after the LH peak, only the granulosa layers located near the basal lamina were stained (Fig. 2B), whereas the layers towards the antrum as well as the cumulus and corona cells were unstained. No staining of the follicular wall was observed at 9-12 (Fig. 2C), 13-15 or 19-21 h after the LH peak. The granulosa cells of the preantral and small antral follicles, the interstitial gland cells, the theca interna cells, and the oocytes in all follicles were unstained.

Nuclear oocyte maturation

The progression of nuclear oocyte maturation relative to the LH peak is presented in Table 2. Before the LH peak all oocytes were at the germinal vesicle stage, i.e. had an intact oocyte nucleus. Germinal vesicle breakdown was at first observed at 5-7 h after LH peak in one third of the oocytes. At 9-12 h the proportion of oocytes at the germinal vesicle breakdown stage and the metaphase I stage was almost identical, and at 13-15 h and 19-21 h, metaphase I and metaphase II were the most frequent stages, respectively.

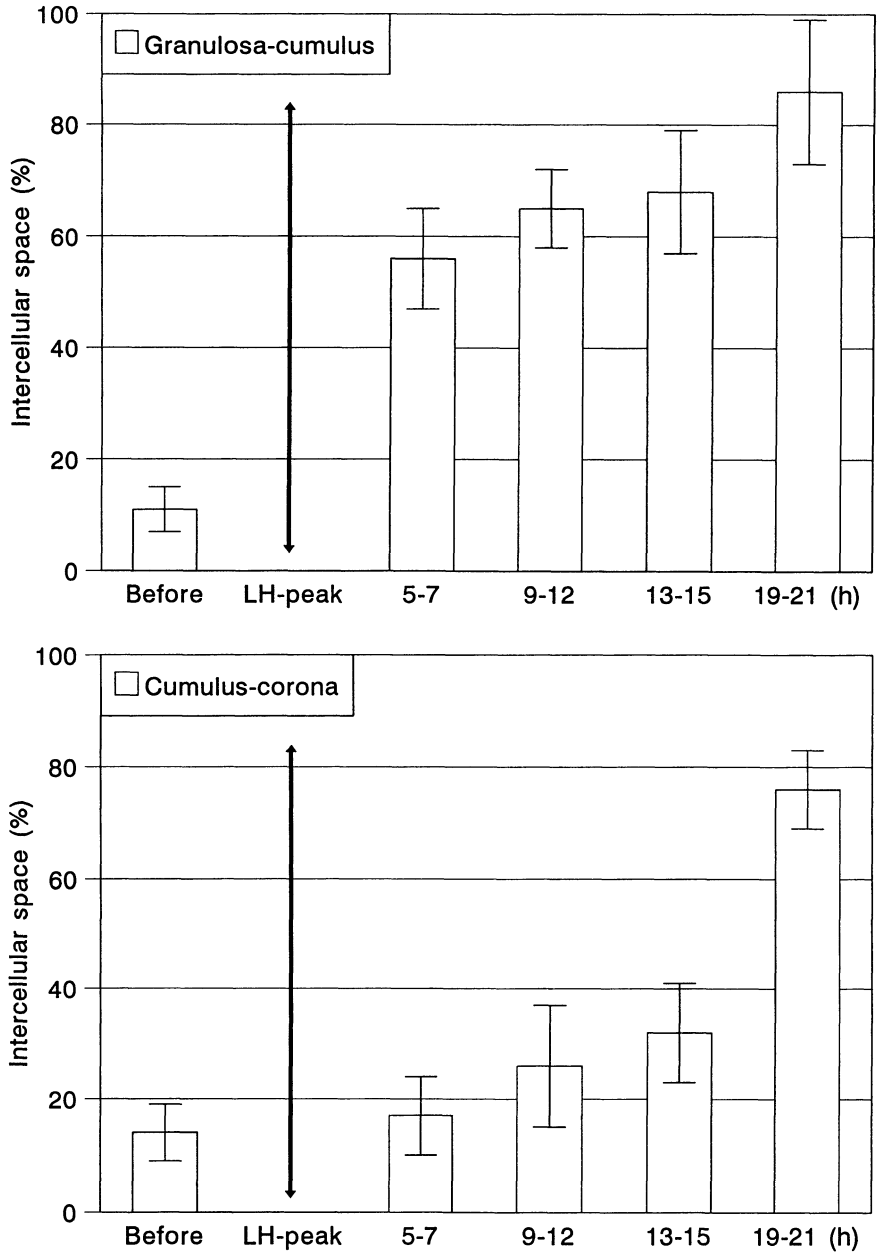

Figure 1 Intercellular spaces between (**A**) granulosa-cumulus stalk cells and (**B**) cumulus-corona cells at different time intervals after the LH peak. The intercellular spaces between the cells of the granulosa-cumulus stalk increased significantly 5-7 h ($p<0.01$) after the LH peak. The intercellular spaces between cumulus-corona cells increased significantly at 13-15 h ($p<0.05$) and again at 19-21 h ($p<0$ 01) after the LH peak.

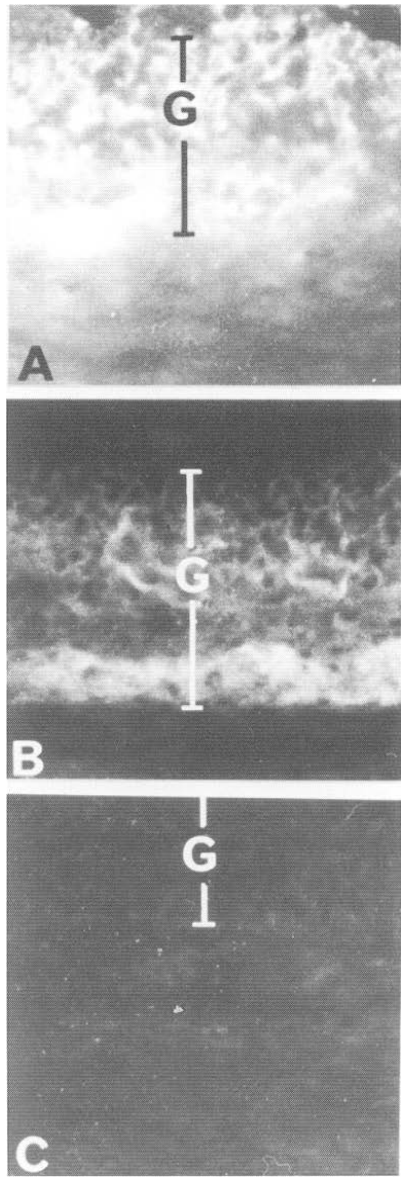

Figure 2. Immunofluorescent staining of aromatase cytochrome P-450 in the wall of superovulated bovine follicles, G granulosa cell layer x230 **A.** Before the LH peak all granulosa cell layers are stained **B.** At 5-7 h after the LH peak staining is restricted to the layers adjacent to the basal lamina **C.** At 9-12 h staining is lacking

Discussion

The fact that fully-grown oocytes undergo spontaneous meiotic maturation after removal from the follicle supports the concept that the follicle cells produce a factor that exerts an inhibitory action on meiotic maturation *(Edwards* 1965, *Biggers* 1972). This inhibitor is either secreted into the follicular fluid or is transmitted directly from the surrounding follicle cells to the oocyte through the extensive network of gap junctions that interconnects the entire granulosa, cumulus, and corona cell population with its enclosed oocyte *(Tsafriri et al.* 1982, *Eppig & Downs* 1984, *Tsafriri & Pomerantz* 1986). It has been suggested that the disruption of the gap junctional pathway between the source of the inhibitor, the membrana granulosa cells, and its target, the oocyte, may be crucial in controling resumption of meiosis *(Gilula et al.* 1978, *Dekel & Beers* 1980, *Dekel et al.* 1981). The present experiment indicates that in superovulated bovine follicles a significant disruption of intercellular communication within the granulosa-cumulus stalk after the LH peak constitutes the functional isolation of the cumulus-oocyte complexes resulting in resumed meiosis. This finding is in agreement with previous results in pig *(Motlik et al.* 1986), rat *(Larsen et al.* 1986, 1987) and sheep *(Murdoch* 1988). It is also in accordance with data published earlier in cattle, where corona-oocyte communication in vivo *(Hyttel et al.* 1986a) as well as in vitro *(Hyttel et al* 1986b, *Hyttel* 1987, *Laurincik et al* 1992 a,b) was maintained at least during the initial resumption of meiosis as indicated by undulations of the nuclear envelope and, in some cases, even up to metaphase I.

The final step in the biosynthesis of estrogens is the aromatization of the androgens, androstenedione and testosterone, to estrone and E2, respectively. Contributions from many laboratories have led to formulation of the "two-cell" model, in which it is postulated that androgen

production occurs in theca cells under LH stimulation, while the subsequent aromatization of the androgens to estrogen is granulosa cell-specific. The latter process is directed by the cytochrome P 450 enzyme aromatase under the control of FSH *(Hsueh et al.* 1984, *Erickson et al.* 1985). Recent immunocytochemical studies have demonstrated predominant localization of aromatase activity in the granulosa cells in the rat *(Yoshinaga-Hirabayashi et al.* 1990, *Ishimura et al.* 1989). The present immunocytochemical study in cattle revealed that aromatase was present only in the granulosa cells of large antral follicles. These data are in accordance with those published earlier in several species including cattle *(Zhong et al.* 1989, *Ishimura et al.* 1989, *Yoshinaga-Hirabayashi et al.* 1990, *Laurincik et al.* 1994). In the present experiment immunostaining was observed in all granulosa cell layers, whereas *Zhong et al.* (1989) only observed staining of the granulosa cells located at the basal lamina in unstimulated cattle. This difference may be due to the superovulatory treatment employed in the present experiment. Moreover, it was clearly demonstrated that the aromatase activity in the granulosa compartment had already decreased at 5-7 h after the LH peak, and a couple of hours later no activity was detectable. These data are in accordance with the decline in the E2 concentrations of the follicular fluid found within the first 6 h after the LH peak *(Dieleman et al.* 1980, *Callesen et al* 1986, *Fortune et al.* 1988, *Hyttel et al.* 1991).

It is concluded that (1) a significant dissociation occurs in the granulosa-cumulus stalk in superovulated bovine follicles within 5-7 h after the LH peak, while dissociation of the cumulus-corona cell layers is not seen until 13-15 h and (2) before the LH peak all granulosa cell layers display aromatase activity, at 5-7 h after the peak only the layers towards the basal lamina are active, and subsequently aromatase activity is absent.

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Sammendrag

Granulosa-cumulus-corona ekspansion og aromataselokalisation i præovulatoriske bovine follikler

Ekspansionen af granulosa-cumulus og corona-cumulus-cellelagene og den follikulære lokalisation af aromatase studeredes i den præovulatoriske periode hos superovuleret kvæg, hvorfra blodprøver opsamledes hver anden time til LH-analyser Ekspansionsprocessen studeredes ved transmissions-

elektronmikroskopi og elektronisk billedanalyse. Lokalisationen af aromatase, et enzym involveret i østrogensyntesen, blev undersøgt ved immunocytokemisk detektion med antistof mod aromatase cytochrome P-450 fra den humane placenta Oocyternes meiotiske modning studeredes ved aceto:orcein-farvning Ekspansion af granulosa-cumuluscelletilhæftningen optrådte i perioden op til 5-7 timer efter LH-toppen (den højeste LH-koncentration målt i løbet af LH-bølgen), hvilket var før nedbrydningen af germinalvesiklen (oocytkernen). Ekspansion af cumulus-corona-cellelagene optrådte derimod først 13-15 og specielt 19-21 timer efter LH-toppen. Før LH-toppen udviste alle follikiens granulosacellelag immunfarvning for aromatase. Fem timer efter LH-toppen udviste kun granulosacellelagene tæt ved basalmembranen farvning, og alle granulosaceller i præparater opsamlet herefter udviste ingen farvning. Ligeledes var granulosacellerne i primære og sekundære follikler, og cellerne i theca interna såvel som alle oocyter ufarvede.

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