Chapter 2

Effects of oFSH stimulation on steroid concentrations in the fluid of preovulatory follicles during final maturation in relation to the LH surge in the cow

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Abstract

FSH-stimulation is used to collect in-vivo oocytes to study maturation and embryonic development. However, not all oocytes are competent. Therefore, selection of functional follicles is a prerequisite. Steroid concentrations in the follicular fluid known to characterize normal preovulatory follicles are not yet defined for FSH-stimulated follicles during the 24 h prior to ovulation. We evaluated the steroid concentrations in all follicles ≥ 5 mm at 2 h before, 6 and 22 h after LH in cows (n=26) treated with oFSH plus GnRH-controlled LH peak. In small follicles (n=101) the concentrations were significantly different from those in large follicles > 8 mm (n=352). In large follicles estradiol 17β was high before LH and decreased thereafter, and progesterone increased at 6 h and further at 22 h after LH, which resembled the pattern in normal preovulatory follicles. Estradiol and androstenedione were distinctly lower before and at 6 h after LH compared to normal follicles or after eCG-stimulation, which could be due to low LH-activity of the oFSH.

Comparison of steroid concentrations in large vs. small follicles indicated that selection criteria for follicles > 9 mm can be limited to: estradiol before LH (> 0.9 µmol/L) and at 6 h after LH (> 0.5 µmol/L), and to progesterone at 22 h after LH (> 0.5 µmol/L). Disqualifying also the few follicles with proper steroid profile but deviating cumulus-oocyte-complex, 51% of the follicles > 9 mm could be considered to enclose a competent oocyte. The stimulation protocol with oFSH and controlled LH surge facilitates the collection of 6 to 7 competent oocytes per cow at specific stages of final maturation.

Keywords: FSH stimulation, final maturation, steroid concentration, oocyte competence, follicle quality

Introduction

Ovarian stimulation protocols are widely used to unravel the molecular and biological mechanisms defining the competence of cow oocytes to develop into viable embryos [1-3]. But not all such oocytes are of equivalent quality. In contrast, in normal cyclic cows, usually only one growing dominant follicle acquires specific structural and functional characteristics to differentiate to the preovulatory stage. During this stage, when a certain level of estradiol 17β is reached in the circulation produced by the growing follicles, the feedback of estradiol 17β on gonadotropins is changed from negative to positive resulting in the preovulatory LH surge that elicits the resumption of meiotic maturation of the oocyte. Six hours after the maximum of the LH surge, concomitant with germinal vesicle break down (GVBD) the estradiol 17β concentration decreases rapidly in the microenvironment of the maturing oocytes and the follicle turns to a progesterone producing structure [4,5]. Only at the completion of a varied set of intracellular changes, including a decrease in the size of Golgi complexes, undulation of the nuclear membrane and a more superficial location of the clusters of cortical granules, the oocyte can finally acquire its full capacity to support fertilization and embryogenesis [6].

Exogenous gonadotropins are used to stimulate development of multiple preovulatory follicles in the mammalian ovary [7] for the collection of increased numbers of oocytes. However, not all oocytes will show the same developmental competence due to deviations in preovulatory follicular development [8, 9]. This heterogeneity in quality is probably due to intrinsic differences between oocytes originating from different follicular microenvironment as can be inferred from the considerable evidence for endocrine regulation changes after stimulation compared to normal cyclic cows. Firstly, a reduction occurs of endogenous basal secretion, pulse frequency and amplitude of FSH and of pulse frequency of LH by more than 50% [10,11], as well as a shortening of the period of preovulatory follicular development from 61 to 41 h in comparison to unstimulated cows [12]. Secondly, superovulation treatment has been shown to induce abnormal amounts of steroids in serum compared to the physiological levels seen during natural cycles [12-16]. Thirdly, different studies have also shown that follicular cells derived from stimulated cows have altered gonadotropin receptor mRNAs [17] and altered abundance of several transcripts of steroidogenic enzymes genes [18].

To explain and to improve the variability in oocyte competence to develop into viable embryos, the amount of required LH bioactivity in the follicle stimulating gonadotropin has been studied extensively. Equine chorionic gonadotropin (eCG) and gonadotropins with high LH bioactivity have been shown effectively to induce multiple follicle development, final oocyte maturation, ovulation and corpus luteum formation [19-21]. However, eCG has an extended half life and can be still detected in serum days after the end of the treatment [12]. Continued support of the follicular cells by eCG increases steroid levels in follicular fluid [22] and therefore prolongs the exposure of the oocyte to estradiol 17β which has been shown to be detrimental to spindle formation in vitro [23,24]. Currently, in clinical applications with purified pituitary FSH is used either with added LH to a bioactivity ratio of 1:1 or with only a low remaining LH bioactivity, both products showing similar yields of viable embryos. Stimulation with FSH with low LH is now more common and has been proven to be an effective alternative to eCG protocols in terms of embryo quality [25-27]. However, in contrast to eCG, this type of FSH results in lower concentrations of estradiol 17β in serum and follicular fluid, and of progesterone in serum [16, 28]. When LH bioactivity is completely absent upon stimulation as with human recombinant FSH, development of preovulatory follicles still takes place but these follicles have a markedly reduced estradiol 17β concentration and contain oocytes that lack cytoplasmic maturation shortly before ovulation [29]. Therefore, balanced amounts of both FSH and LH are required for proper stimulation of follicles in the cow. The resulting steroid levels in the follicular fluid appear to be important determinants in the production and complex interaction of growth factors ensuring follicle growth and differentiation, and ultimately oocyte nuclear and cytoplasmic maturation [30, 31].

The developing ovarian follicle is one of the most rapidly proliferating normal tissues known in vivo, and granulosa cells account for the majority of this follicle expansion. During the preovulatory period a large change in the steroid content occurs concomitant with this proliferation [32]. Since superovulation leads to varying degrees of preovulatory follicle size, changes in steroid concentration may also arise in these follicles that most likely concur with varying degrees of oocyte developmental competence. Recently, a significant relationship has been reported during GnRH induced ovulation between follicle size, serum progesterone and pregnancy rates [33]. However, in cows stimulated with exogenous gonadotropins, serum steroid levels reflect a sum of steroid produced by many follicles. As a result, predicting oocyte quality based on serum steroid concentration is not possible. In striking contrast to the wealth of information available regarding superovulatory response and embryo quality

after treatment with FSH, little is known concerning steroid concentrations in preovulatory follicles after FSH treatment in relation to size of the follicles. Therefore, follicular fluid concentrations of estradiol 17β, progesterone, testosterone and androstenedione were studied in cows stimulated with FSH with low remaining LH activity at onset, after initiation and at completion of final maturation of the oocyte.

2. Materials and methods

2.1. Experimental design

Normally cyclic Holstein-Friesian cows were treated for superovulation using the protocol as described before [34] with oFSH and a Crestar/GnRH-controlled LH surge (Fig. 1). The concentration of LH in plasma was monitored to determine the time of ovariectomy for the collection of follicles relative to the maximum of the LH surge, and to exclude the few, eventual cows with deviating LH profiles [35]. Cows were allocated at random to three experimental groups: 1) at onset (2 h before LH), 2) after initiation (6 h after LH), and 3) at completion (22 h after LH) of final maturation. The experiment was carried out as approved by the Ethical Committee of the Veterinary Faculty of Utrecht University. For comparison of steroid levels, data was used that had been collected previously for preovulatory follicles from eCG-stimulated cows [36] and from untreated, cyclic cows [37].

2.2. Animals and treatments

For the FSH group, thirty-three lactating Holstein-Friesian cows were selected after clinical examination, that were on average 4.4 y of age and 185 d post partum. While on pasture, progesterone concentrations in peripheral blood samples were measured three times a week. After at least four weeks, cows (n=30) with normal cycles were pre-synchronized preceding the experiments in groups of 4 animals using an ear implant for 9 days and prostaglandin as described before [34]. Following the synchronized estrus, the cows were housed inside, and were fed silage and concentrate with water supplied ad libitum. On Day 9 of the estrous cycle (Day 0 is estrus), the cows received another ear implant (3 mg norgestomet, Crestar; Intervet International B.V., Boxmeer, The Netherlands) for 5 d but not further combined with norgestomet and estradiol valerate im. From Day 10 onwards, the cows were treated with oFSH im (Ovagen ICP, Auckland, New Zealand) twice a day in decreasing doses during 4 d (3.5, 2.5, 1.5 and 1 mL; in total 17 mL equivalent to 299 IU NIH-FSH-S1). Prostaglandin (PG; 22.5 mg

Prosolvin im; Intervet International B.V.) was administered together with the fifth dose of FSH. Ear implants were removed at 49 or 50 h after PG, and GnRH (0.021 mg Receptal im; Intervet International B.V.) was administered concomitantly with the 50 h after PG implant removal to induce the LH surge. Follicular fluids were collected following ovariectomy (OVX) at 50, 58 and 74 h after PG corresponding with 2 h before, 6 and 22 h after the maximum of the LH surge, respectively.

For the eCG group, pre-synchronized cows in a previous experiment had been treated with 3,000 iu eCG (Folligon im; Intervet International B.V.) on Day 10 and 22.5 mg PG 48 h later on Day 12 [36]. Follicular fluids had been collected by transvaginal ultrasound-guided puncture from preovulatory-sized follicles at 12 h before, 4, 12 and 22 h after the maximum of the endogenous LH surge.

For the untreated, cyclic group, pre-synchronized cows in a previous experiment had been monitored for the onset of luteolysis by frequently determining the concentration of progesterone in peripheral blood from Day 14 onwards [37]. Preovulatory follicular fluids had been collected following OVX at 48 to 62 h after luteolysis while, in cows, the interval between onset of luteolysis and LH surge is 61 h [38].

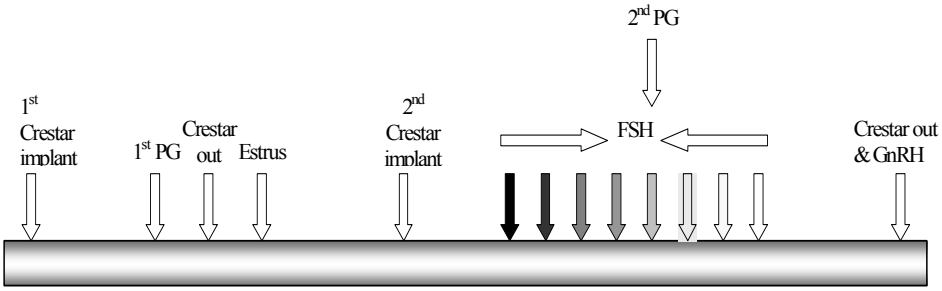

Figure 1. **Schedule of treatment for pre-synchronization and superovulation with a Crestar/GnRH-controlled LH surge to obtain oocytes at specific times of development;** PG=prostaglandin. The precise timing of the administration of PG during the FSH treatment was determined at intervals of 1 h to allow for periods of 1 h between each cow at ovariectomy. Similarly, removal of the 2nd ear implant (Crestar out) and administration of GnRH were carried out at 1 h intervals; a maximum of 4 cows was used every treatment run.

2.3. Blood sampling

Heparinized blood samples were collected from the jugular vein every day during the experimental cycle, every 3 h starting 12 h before removal of the second implant and every hour thereafter for 6 h. After immediate centrifugation at 4°C, plasma was stored at -25°C.

2.4. Collection of follicles

For every treatment run with a group of 4 cows OVX was performed at 1 h intervals (See text of Fig. 1) by laparotomy through flank incision under local infiltration anesthesia [5]. Ovaries were collected in 0.9% NaCl at 37°C and immediately transported to the laboratory. The numbers of follicles were recorded per size class 2 to 5, 5 to 8 and > 8 mm. The content of each follicle > 8 mm was aspirated using an 18-ga winged infusion set needle attached to 15 ml polystyrene conical tube under low pressure by means of a suction pump, and were then immediately stored on ice at 4°C. The size of the follicles was calculated from the volume of follicular fluid after collection which resulted in a slight underestimation due to some eventual loss of fluid and not accounting for the thickness of the follicular wall. As can be derived from the formula for volume (= 4/3*pi*r3 with 2r=diameter) underestimation is more manifest with small follicles. Follicles with a calculated diameter of > 9 mm can be assumed to have been > 10 mm. After retrieval of the cumulus oocyte complexes (COC) under a stereo microscope, the follicular fluids were centrifuged 3,000 *g* for 10 min at 4°C and stored at -25°C until analysis for steroids. The morphological appearance of COCs at the successive stages of in vivo maturation was rather different from that of slaughterhouse oocytes. Therefore, the standard qualification as used for in vitro oocytes was not applied to distinguish between grades. The COCs were further processed and stored for other studies into the transcriptome of oocyte and cumulus. The fluid of follicles of 5 to 8 mm as established by ruler was aspirated by a 2 ml syringe to estimate the volume, and was stored at -25°C until analysis for steroids.

2.5 Assays for hormones in plasma and follicular fluid

Concentrations of LH were determined in duplicate aliquots of 100 μL plasma by a homologous double-antibody RIA as described previously [4]. Bovine LH (bLH-7981) was used for iodination and standards, and rabbit anti-bLH (8101) as antiserum. Specificity of the RIA was high as indicated by low cross-reactivity for other pituitary hormones and by the observed parallelism with NIH-LH-B4 and NIH-LH-B9 for the range of 0.2 to 50 μg/L. The limit of quantitation was 0.2 μg/L LH.

Concentrations of progesterone in plasma were determined in duplicate aliquots of 100 μL by solid-phase ^{125}I RIA method (Coat-A-Count TKPG; Diagnostic Products Corporation, Los Angeles, CA, U.S.A.) as validated previously [39]. The limit of quantitation was 0.8 nmol/L progesterone.

Concentrations of steroid hormones in follicular fluid were determined in aliquots of 1 to 25 µL fluid dependent of the hormone and the size of the follicle by solid-phase ^{125}I RIA methods (Coat-A-Count, Diagnostic Products Corporation; progesterone (P): TKPG; estradiol 17β (E): TKE2; testosterone (T): TKTT ; androstenedione (A): TKAN) as validated for blood plasma of cows [39] with slight modifications. Briefly, the follicular fluid samples were extracted with 2 mL freshly opened diethyl ether (BDH Laboratory Supplies, Poole, England). To determine and correct for the efficiency of extraction approx. 10,000 dpm ^{3}H-steroid were added before extraction to a separate parallel series of similar aliquots of fluid. After evaporation of the organic solvent the samples and efficiency series were dissolved in 250 µL borate buffer (for E) or in 250 µL zero plasma of the manufacturer. Duplicate volumes of the samples were then incubated in the antibody coated tubes (Coat-A-Count). Specificity of the RIAs was high as indicated by low cross-reactivity for other steroid hormones of physiological importance, for details see the manufacturer's manual. (Coat-A-Count). The limits of quantitation were 8, 5 to 92, 4, and 5 to 13 nmol/L dependent of the sample volume used and the standard series applied for P, E, T and A, respectively.
Calculation of all hormone results was done applying the spline approximation for the standard series from RIASmart (Packard Instruments Company, Meriden, CT, U.S.A.). The calculated doses were < 4 % different from the defined doses over the entire range. In general, the intra- and inter-assay coefficients of variation were < 10 and < 15% for all assays, respectively.

2.6. Statistics

Results are presented as means ± SEM, and data were analyzed by using SPSS statistical package, version 12.0 for Windows (SPSS Inc., Chicago, IL). Results that were not normally distributed were log-transformed before analysis. Concentrations of follicular fluid steroid between different follicle sizes were analyzed by the linear mixed model procedure including follicle size as fixed effect and the individual cow as a random effect. Bonferroni correction was used when significance tests were performed. Bivariate correlation analysis (calculation of Spearman's coefficient) was used to assess the correlation between follicle size and follicle steroid content on raw data. A *P* value less than 0.05 was considered significant.

3. Results

Data of four cows were not included, two had no proper LH suppression during Crestar treatment, and two showed < 3 preovulatory-sized follicles (POF) with a diameter 10 mm. The average number of POF was 8.6, 13.0 and 10.4 per cow of the 2 h pre LH, 6 h and 22 h after LH group, respectively. The follicles were subdivided into the size categories small: 5 to 8 mm, and large: > 8 to 10, > 10 to 12 and > 12 to 16 mm as calculated from the volume of follicular fluid. No significant differences were observed between the 3 groups regarding the number of follicles per size category (Table 1). A total of 429 follicles (≥ 5 and ≤ 16 mm) were analyzed, 150 follicles 2 h pre LH, 157 follicles 6 h and 122 follicles 22 h after LH, respectively. Variable numbers of 2 to 5 mm follicles were recorded (mean: 21 per cow).

Table 1. Average number of follicles per size category per cow at different times preceding ovulation in cows treated with an oFSH protocol for superovulation with a controlled LH surge.

Time (h) relative to LH peak (n cows)	Size category (mm)[1]				
	> 5 to 8	> 8 to 10	> 10 to 12	> 12 to 16	> 16
-2 (10)	3.9	4.7	3.3	3.4	0.6
6 (8)	5.0	4.3	5.9	4.6	0.6
22 (8)	2.8	3.6	5.3	3.6	0.8

[1] size calculated from collected follicular fluid volume.

3.1. Estradiol 17β

The concentration of estradiol 17β was highest in the fluid of follicles > 8 mm at onset of final maturation that is 2 h pre LH, compared to the level at later stages after the LH peak (Fig. 2). The concentrations in the three larger size categories were not significantly different but all higher ($P < 0.05$) than in 5 to 8 mm follicles. The average level (1.41 ± 0.10 μmol/L, n=113) in follicles sized > 8 to 16 mm of the pre LH group treated with oFSH was significantly ($P < 0.05$) lower than that found for pre LH follicles following eCG treatment (4.78 ± 0.23 μmol/L, n=86) and also than the level in the preovulatory follicle of untreated cyclic cows just before the LH peak (4.25 ± 0.30 μmol/L, n=11); only the average (4.46 ± 0.43 μmol/L, n=5) of the highest five of the

oFSH group was similar to that of the other treatment groups. At the later stages during final maturation, at 6 and 22 h after LH, the differences between the concentrations of estradiol 17β in the fluid of the different size categories remained, with the level in the follicles sized 5 to 8 mm being significantly lower ($P < 0.05$) than that in the other categories. A significant correlation between size and concentration of estradiol 17β was observed 6 h after LH.

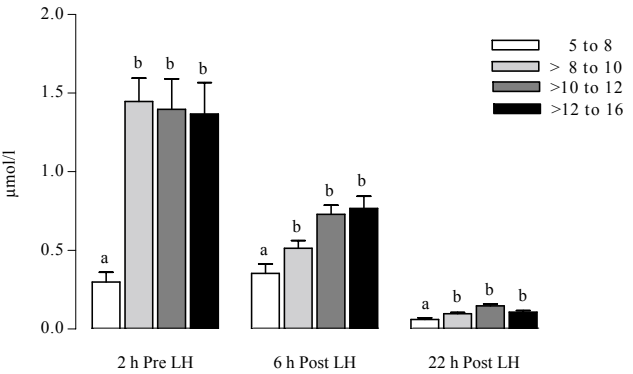

Figure 2. Estradiol 17β concentrations in the fluid of follicles collected during final maturation relative to the maximum of the controlled LH surge in oFSH treated cows; concentrations are mean ± SEM, indices a,b indicate significant differences within a time group.

3.2. Progesterone

The concentration of progesterone in the fluid of the different size categories varied considerably during final maturation (Fig. 3). In the pre LH group, the progesterone concentration was at a similar and low level in all follicles which was significantly different from that found in preovulatory follicles of untreated cyclic cows (0.26 ± 0.07 µmol/L, n=11) but the level in both groups was lower ($P < 0.05$) than that in pre LH follicles following eCG treatment (0.34 ± 0.08 µmol/L, n=86). At 6 h after LH the progesterone concentration increased in particular in follicles > 10 mm which was similar to the increase found after eCG treatment (0.46 ± 0.05 µmol/L, n=59; at 4 h after LH). Shortly before ovulation the highest level was observed in the largest category of follicles (> 12 to 16 mm; 0.89 ± 0.17 µmol/L, n=29) which was higher than that found after eCG treatment at 22 h after LH (0.54 ± 0.04 µmol/L, n=108), but the level in the follicles > 8 to 12 mm was similar to that after eCG treatment. A significant correlation

between size and concentration of progesterone was observed 6 and 22 h after LH that is in the period of initiation and at completion of maturation.

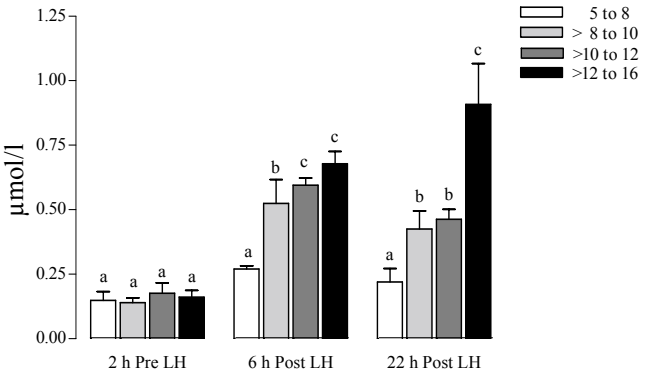

Figure 3. Progesterone concentrations in the fluid of follicles collected during final maturation relative to the maximum of the controlled LH surge in oFSH treated cows; concentrations are mean ± SEM, indices a,b,c indicate significant differences within a time group.

3.3. Testosterone

The most marked changes in the concentration of testosterone occurred in the small follicles of 5 to 8 mm (Fig. 4). At all stages of final maturation these levels were significantly ($P < 0.05$) higher than that of the other larger size categories in which a significantly higher ($P < 0.05$) level was observed in > 8 to 10 mm follicles at pre LH and 6 h after LH. At all respective stages during final maturation a significant correlation was observed between size and concentration of testosterone. The molar ratio of estradiol 17β over testosterone concentrations showed a significant correlation with size of the follicles in the pre LH group being 20.8 ± 4.3 (n=32), 36.7 ± 4.6 (n=45) and 57.9 ± 8.2 (n=32) (average ± SEM) for > 8 to 10, > 10 to 12 and > 12 to 16 mm diameter, respectively. A similar increase with size but at lower values was observed for the 6 h post LH group.

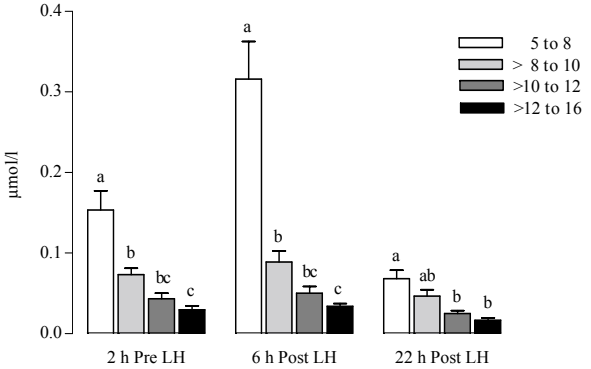

Figure 4. Testosterone concentrations in the fluid of follicles collected during final maturation relative to the maximum of the controlled LH surge in oFSH treated cows; concentrations are mean ± SEM, indices a,b,c indicate significant differences within a time group.

3.4. Androstenedione

The concentration of androstenedione varied between small vs. large follicles but no significant differences were observed between the large size categories of pre LH or 6 h after LH follicles (Fig. 5). The levels of androstenedione at 22 h after LH were not measured since this steroid can be considered to play no role at the stage of completion of maturation. The average level in large follicles > 8 to 16 mm before LH (0.11 ± 0.01 µmol/L, n=104) was markedly lower ($P < 0.05$) than that in follicles of eCG treated and of untreated normal cyclic cows at the corresponding stage (0.83 ± 0.09 µmol/L, n=86 and 1.33 ± 0.20 µmol/L, n=11, respectively). At 6 h after LH the average level in large follicles was the same as before LH (0.11 ± 0.01 µmol/L, n=115) which was still lower ($P < 0.05$) than that following eCG despite the obvious decrease in the latter group (0.58 ± 0.06 µmol/L, n=62). A significant correlation was observed between size and concentration of androstenedione for follicles at 6 h after LH.

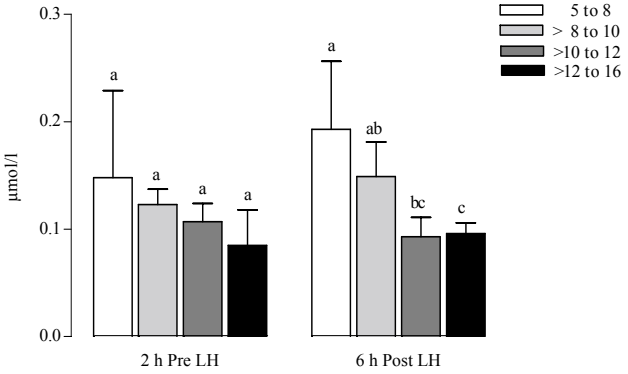

Figure 5. Androstenedione concentrations in the fluid of follicles collected during the first two stages of final maturation relative to the maximum of the controlled LH surge in oFSH treated cows; concentrations are mean ± SEM, indices a,b,c indicate significant differences within a time group.

3.5. Threshold steroid levels for large follicles

To discriminate between follicles being functional *vs.* non-functional it was assumed that the normal pattern of changes of steroid concentrations occurred only in healthy preovulatory sized oFSH stimulated follicles. Therefore, the concentrations of steroids in small 5 to 8 mm follicles were used as reference. The threshold value for size of preovulatory follicles was set at > 9 mm as calculated from the volume, since at about this size follicles either develop further or undergo atresia.

In the pre LH group 18/122 (: 15%) of the large follicles were > 8 to 9 mm. Taking then the estradiol 17β (E) concentration + 2SD of small follicles as threshold value (Fig. 6) 38/104 (: 36.5%) of the follicles > 9 mm showed a lower E concentration. All of the remaining large follicles (n=66) met the criteria derived from the small follicles for progesterone (P) and testosterone (T). In the few follicles with a too high P concentration this deviation always coincided with an E concentration below the threshold value.

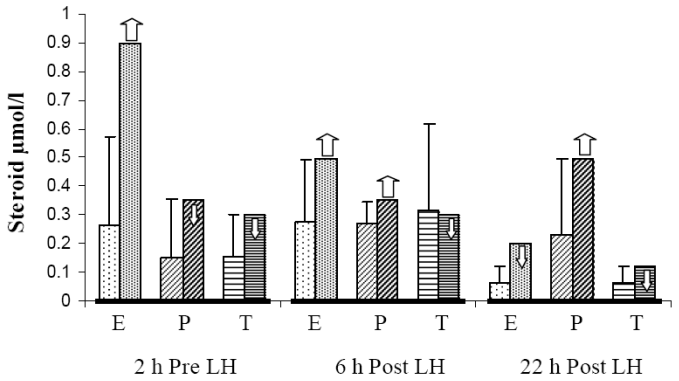

Figure 6. Threshold levels of steroid concentrations in the fluid of follicles collected during final maturation relative to the maximum of the controlled LH surge in oFSH treated cows; E: estradiol, P: progesterone, T: testosterone. Of each pair of bars, the left bars represent the mean ± SD steroid concentration for follicles 5 to 8 mm, and the right bars are the threshold value for large preovulatory follicles; arrows pointing up- or downwards indicate whether the threshold values are lower or upper limits for the qualification functional, respectively.

In the 6 h post LH group 11/123 (: 9%) of the large follicles were > 8 to 9 mm. Taking again the E concentration of the small follicles but now + 1SD as criterion in view of the rapidly decreasing E level at this stage of maturation, 47/112 (: 42%) of the follicles > 9 mm were below the threshold value (Fig. 6). Similar to the findings in the pre LH group all remaining large follicles (n=65) met the criteria for P and T concentrations. In the few follicles with a too low P concentration and in the 9/112 follicles with an exceptionally high P (1.95 µmol/L) this deviation always coincided with an E concentration below the threshold value.

In the 22 h post LH group 15/107 (: 14%) of the large follicles were > 8 to 9 mm. Taking now the P concentration of the small follicles + 1SD as criterion in view of the stage of completion of final maturation, 36/92 (:39%) of the follicles > 9 mm were below the threshold value (Fig. 6). All remaining large follicles (n=56) met the criteria for E and T concentrations.

4. Discussion

In the preovulatory follicle of the normal cyclic cow the successive stages of final follicular and oocyte maturation are characterized by the concentrations of steroids in the follicular fluid [5,40]. Before the LH surge before onset of maturation estradiol 17β is predominant while the oocyte is still at the germinal vesicle (GV) stage. Coinciding with the LH peak the level of progesterone in the normal preovulatory follicle shows a temporary increase lasting about 6 h [5], and at 6 h the level of estradiol although decreasing is still high while the oocyte is undergoing GVBD. At completion of maturation at 22 h after the LH peak progesterone is predominant and the oocyte is at metaphase II showing also cytoplasmic maturation [6]. After oFSH stimulation the relative pattern of changes with regard to estradiol and progesterone concentrations in large follicles is in agreement with that in the normal preovulatory follicle. However, before the LH surge the concentration of estradiol is distinctly lower also in comparison to follicles stimulated with eCG. Even when the follicles with an estradiol level below the arbitrary threshold are excluded the average concentration (2.08 μmol/L) is only about half of that in the normal untreated preovulatory follicle. Before the LH surge, estradiol levels in the preovulatory follicle depend on the capacity of theca cells that have acquired LH binding sites to respond to LH with increased androstenedione production, and also to the ability of granulosa cells to convert this androgen to estradiol [41]. The significantly lower androstenedione concentration in the oFSH stimulated large follicles in comparison to that of the normal cyclic preovulatory follicle indicate that the low estradiol levels could be due to the low LH bio-activity of the oFSH preparation used. Treatment with eCG having a high LH bio-activity resulted in higher androstenedione and consequent estradiol concentrations.

Synthesis of estradiol and progesterone is regulated by the relative levels of steroidogenic enzymes of the delta 4 (for P) and 5 (for E) pathways expressed in theca and granulosa cells. The precursor for both pathways pregnenolone, is produced in the mitochondria from cholesterol that is mobilized from the peripheral blood [42]. Since the progesterone levels in the fluid of the oFSH stimulated large follicles were comparable to those in the normal cyclic preovulatory follicle in particular at 6 h after LH [5] contrary to the levels of estradiol, it is unlikely that the reduced estradiol concentration in oFSH stimulated follicles at onset of final maturation is due to a defect in cholesterol mobilization or synthesis of pregnenolone. Further in the delta 5 pathway, P450 17α-hydroxylase ($P450_{17\alpha}$) which is expressed exclusively in theca cells [43] and 3β-hydroxysteroid dehydrogenase (3βHSD) are responsible for converting

pregnenolone into androstenedione. Soumano et al. [18] have shown that eCG and FSH treatments have different effects upon follicular $P450_{17\alpha}$, but not upon 3βHSD and P450 aromatase mRNA abundance. The finding that 5 to 8 mm follicles contain higher concentrations of testosterone and androstenedione, and the lack of this accumulation in oFSH stimulated large follicles strongly indicate an active aromatase enzyme complex. In summary, it can be concluded that the low estradiol level is due to a reduced activation of $P450_{17\alpha}$ as a result of low LH activity in the oFSH.

Despite the significantly lower estradiol level in the large follicles at 2 h before the LH surge a substantial proportion of the oocytes will be developmentally competent. Previous studies applying the same stimulation protocol with oFSH and a controlled LH surge have demonstrated that a proportion of 43% of such oocytes develop in vitro to morula and blastocyst stages [1] and in vivo result in embryos with a normal incidence of apoptotic cells [44] and a low proportion of cells with chromosomal aberrations [45].

Although it is known that in the periovulatory period, ovulation and luteinization are dependent on local progesterone actions [46], little information is available regarding the role of progesterone in mammalian oocyte maturation and developmental competence. Recently, inhibition of LH induced progesterone production by granulosa and cumulus cells in Rhesus monkeys in vivo [47] or during IVM of porcine cumulus oocyte complexes [48] resulted in suppression of GVBD suggesting an important role for progesterone in meiotic resumption. Interestingly, in the present study, when final maturation was initiated at 6 h after LH, the concentration of progesterone in follicles > 10 to 16 mm was significantly higher than in those < 10 mm while with completion of maturation at 22 h after LH the follicles > 12 mm showed a sharp increase of the progesterone level. This latter increase likely reflects increased numbers or more differentiated granulosa cells. A significant effect of follicle size during GnRH induced ovulation on pregnancy status has recently been reported [33]. Follicles ovulated at size < 12.8 mm showed a decrease in the ability to support the pregnancy until day 60. Whether this is related only to a reduced function of the corpus luteum originating from such follicles or also to oocyte competence is not clear. It can be speculated that larger follicles with higher progesterone levels have an increased chance to contain competent oocytes. The higher level of progesterone is probably due to a rise of LH receptors in the follicle. A substantial increase of LHr mRNA has been reported when follicle size increases from > 10 to 15 mm [49].

4.1. Selection of oFSH stimulated follicles

It can be assumed that competent oocytes are primarily enclosed by functional preovulatory-sized follicles showing the normal changes in steroid concentrations. To distinguish such follicles after oFSH stimulation, criteria were developed on the basis of the steroid concentrations in the follicular fluid dependent of the stage of final maturation. In view of the reduced estradiol level at 2 h before LH in comparison to that in the normal preovulatory follicle the criteria were derived from the steroid levels in the group of non-responding (or still growing) 5 to 8 mm follicles. During the un-stimulated cycle deviation that is the beginning of the difference in growth rate between the two largest follicles, begins at an average diameter of 8.5 mm [50] and is associated with a differentiation of the concentration of estradiol [51]. This suggests that the functionality of the follicles sized > 8 to 9 mm following the oFSH treatment may be critical. Indeed, this category showed a 65% chance of not meeting the criteria. When the follicles > 8 to 9 mm were excluded for selection, a 52 to 54% of the follicles > 9 mm showed a proper steroid profile at the respective stages of maturation. Few oocytes (16/352) were observed with a (huge) expansion of the cumulus at 2 h before and at 6 h after LH, and without expansion at 22 h after LH. Half of these deviating oocytes were found in follicles not meeting the steroid criteria and the other half in follicles considered to be functional (8/187). Disqualifying also these follicles resulted in an average 51% (179/352) of functional follicles after oFSH stimulation with a controlled LH surge. This proportion was not correlated with size for follicles >9 to 16 mm (data not shown). The similarity of the proportion of functional follicles with that of viable embryos in embryo transfer programmes using the same oFSH (60%; [52]) strongly suggests that these follicles enclose developmentally competent oocytes. Although it has been suggested for human oocytes that developmental competence is related more closely to the ratio estrogen to androgen [53] and a positive correlation of this ratio with follicular size was observed in the groups before and at 6 h after LH, the set of criteria to select follicles can be limited to the concentration of estradiol before and at 6 h after LH, and of progesterone at 22 h after LH.

In conclusion, the stimulation protocol with oFSH and controlled LH surge facilitates the collection of 6 to 7 competent oocytes per cow at specific stages of final maturation. In view of the similarity between early embryonic development in the cow and the human regarding the need for maternally derived molecules during the first cell cycles after fertilization, the data presented here will provide important reference points

for collecting oocytes to study the transcriptome in relation to developmental competence.

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