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Ultrastructure of Bovine Ovarian Follicles Induced to Extended Growth by Perioestrous Suprabasal Progesterone Levels

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[1]Department of Obstetrics and Gynaecology and [2]Department of Clinical Chemistry, Swedish University of Agricultural Sciences, Uppsala, and [3]Swedish Association for Livestock Breeding and Production, Eskilstuna, Sweden.

Duchens, M., H. Rodríguez-Martínez, M. Forsberg and H. Gustafsson: Ultrastructure of bovine ovarian follicles induced to extended growth by perioestrous suprabasal progesterone levels. Acta vet. scand. 1996, 37, 239-250. The present study was undertaken to determine if a short-term prolonged growth of the ovulatory follicle (12 to 18 h after expected time of ovulation), induced by progesterone implants, would cause ultrastructural changes in the follicular wall. Oestrous behaviour, follicular growth, follicular and blood plasma levels of oestradiol-17ß, progesterone and plasma luteinizing hormone (LH) were monitored in heifers oophorectomized at 9 to 12 h (controls) or 36 h after the onset of oestrus, in order to sample the pre-ovulatory follicle present. The suprabasal plasma progesterone concentrations (approximately 1.2 nmol L^{-1}) allowed expression of oestrus at the expected time, but ovulation was delayed owing to the absence of a LH-surge. The resulting prolongation of follicle growth was associated with mild degenerative changes in the follicle wall, i.e. both granulosa and thecal cells presented increased electron density, higher amounts of secondary lysosomes and lipid droplets, increased intercellular spaces with presence of debris. No signs of luteinization were seen.

ovary; follicle wall; electron microscopy; bovine.

Introduction

Administration of progesterone to cycling heifers, resulting in high plasma concentrations of the hormone, extends the oestrous cycle and suppresses oestrus and ovulation *(Christian & Casida* 1948), with a continuous turnover of follicular waves *(Bergfelt et al.* 1991). Low progesterone concentrations after luteolysis (3 to 10 nmol L^{-1} in plasma) extend the life span of the ovulatory follicle and lengthen the oestrous cycle *(Sirois & Fortune* 1990). Normal oestrus and ovulation occur after removal of the exogenous progesterone, but the conception rate after insemination is impaired *(Savio et al.* 1993, *Wehrman et al.* 1993). With progesterone concentrations of 1 to 2 nmol L^{-1}, i.e., just above the basal levels at oestrus, oestrous behaviour is altered or prolonged instead of being suppressed. More specifically, ovulatory follicle growth is prolonged, and oestradiol release is increased, thereby leading to delay in the preovulatory LH-peak and in ovulation *(Duchens et al.* 1994, *Duchens et al.* 1995a).

Many of the disturbances induced by supraba-

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sal concentrations of progesterone, like prolonged oestrus, weak expression of oestrous signs, longer interval between onset of oestrus and the peak of LH, and a delayed ovulation, are similar to those occurring in repeat-breeding cattle *(Bostedt* 1976, *Erb et al.* 1976, *Maurer & Echternkamp* 1982) and particularly observed in repeat-breeding heifers *(Gustafsson et al.* 1986, *Albihn* 1991). It remains to be proven whether these disturbances can be caused by a naturally occurring hormonal asynchrony in repeat breeders. We induced suprabasal progesterone concentrations by inserting subcutaneous implants during prooestrus and used the method as an experimental model to study the endocrine, ovarian and behavioural asynchronies described in repeat-breeding heifers. In a recent study, applying this model, we managed to influence oestrous behaviour and delay ovulation in normal heifers. Despite repeated inseminations from onset of oestrus until ovulation, fertility was impaired *(Duchens et al.* 1995b). The reason/s behind this lowered fertility are yet not fully understood.

The final growth of the ovulatory follicle follows a precise sequence of changes *(McNatty et al.* 1981, *Dieleman et al.* 1983). A subtle alteration of this timed sequence caused by a prolongation of ovulatory follicle growth may influence the functioning of the granulosa/thecal cells, thereby resulting in changes in the microenvironment of the follicle, including the oocyte, which lead in turn to decreased fertility. Ultrastructural examinations describing the morphology of granulosa and theca interna cells during the normal oestrous cycle have been performed in ruminants, including the cow *(Priedkalns & Weber* 1968) and sheep *(O'Shea et al.* 1978, *Cran et al.* 1979). However, descriptions of the follicle wall under conditions of continued growth or under the effect of exogenous progesterone are, to our knowledge, not available.

The objective of the present study was to determine if an experimentally induced, short-term prolongation in the growth of the ovulatory follicle in heifers would cause changes in the morphology of the granulosa/thecal cells.

Materials and methods

Animals and treatments

The experimental design was reviewed and approved by the Ethical Committee for Experimentation with Animals, Sweden. Ovaries with preovulatory follicles were obtained from 4 cycling heifers of the Swedish Red and White and Swedish Friesian breeds. The experimental design is depicted in Fig. 1, consisting of 2 rounds (day 0 = day of the previous ovulation), the heifers being subjected to a sham implant (heifer 1) or treated with progesterone implants (heifers 2-4). The implants, placed subcutaneously in the neck region, were made of silicone elastomer filled with crystalline progesterone (4-pregnene-3,20-dione, Sigma Chemical Co., St. Louis, MO, USA). Each implant contained a dose of 10.6 mg kg^{-1}, which had previously been determined to result in a plasma progesterone concentration at proestrus-oestrus of ~1 nmol L^{-1} *(Duchens et al.* 1995a). In heifer 1 (sham implant, control) and treated heifer 2 (progesterone implant, treatment A), the ovary bearing the dominant follicle was removed 9 to 12 h after onset of standing oestrus. In the remaining 2 heifers (3-4), the ovary was removed 36 h after the onset of standing oestrus (treatment B). In heifers with no standing oestrus, the onset of oestrus was assumed to occur 36 h after the appearance of prooestrus, and it was confirmed by the presence and intensity of secondary signs of oestrus (increased attention, cervical mucus discharge, sinking of the lumbar region at palpation, vulvar mucosal reddening and increased uterine tonus). The unilateral oophorectomy was performed while the ani-

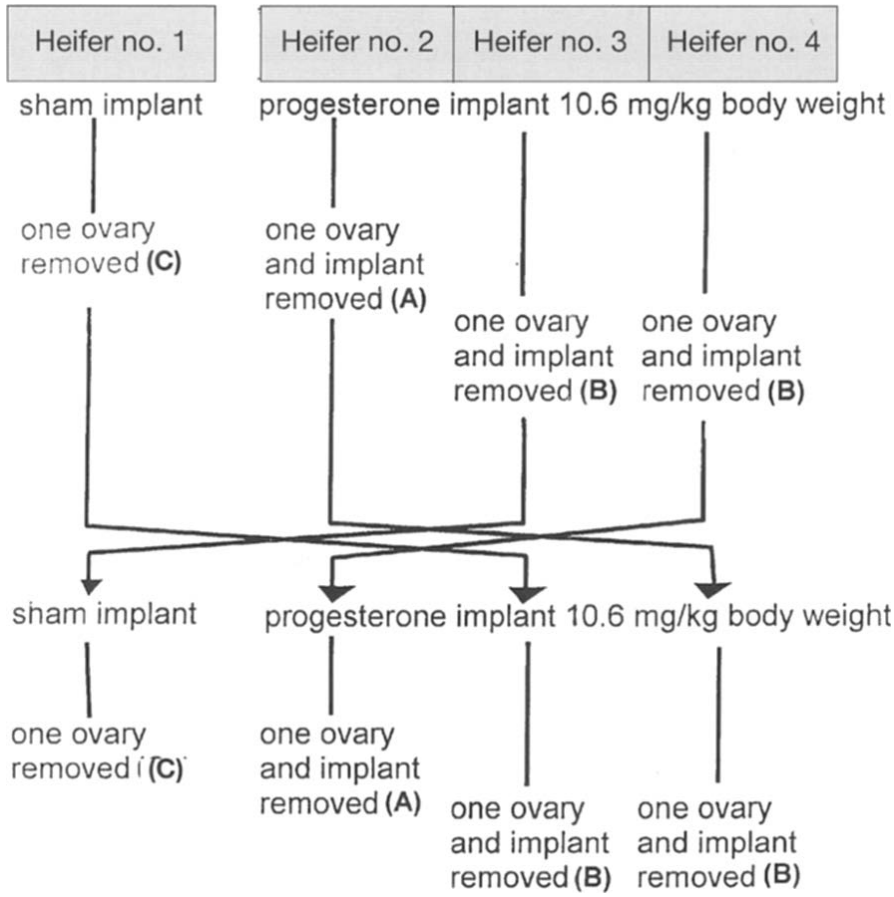

Figure 1: Flow chart depicting the experimental design.

mals were standing. The mesovarium was crushed with an ecrasseur and the ovary removed. The progesterone implants were removed immediately thereafter. After surgery, the heifers were allowed 2 resting oestrous cycles of normal duration. After ovulation in the second resting oestrous cycle, the heifers were again randomly sham (control) or progesterone (treatments A-B) implanted (Fig. 1, second experimental round, prior to second oophorectomy) and the protocol of surgery, oestrous detection and oophorectomy repeated.

Ultrasonography and oestrous detection

Growth of the dominant follicle was monitored at 12-h intervals with a linear array ultrasound scanner (Aloka SSD-210DXII, Aloka Co. Ltd, Japan) equipped with a 7.5 MHz transducer. Oestrous signs were recorded every 6 to 8 h and scored according to their intensity of expression *(Duchens et al.* 1995a). Standing oestrus was controlled every 12 h with a mounting bull.

Morphological examination

Immediately after removal of an ovary, the pre-

ovulatory follicle was punctured with an 18G needle and the follicular content aspirated. The follicle was thereafter washed, using the same needle, with PBS supplemented with fetal calf serum (1% v/v, National Veterinary Institute, Uppsala, Sweden) and kanamycin (25 μg ml^{-1}, Sigma Chemical Co., St. Louis, MO, USA). The follicular wall was thereafter sliced (1 mm in width) and promptly fixed in a solution of 3% glutaraldehyde in cacodylate buffer (500 mOsm, pH 7.2). The follicular fluid and flushings were immediately inspected under a stereomicroscope in search of the oocyte which, when found, was fixed as described above. The follicular fluid was frozen at –20°C until assayed for oestradiol-17β and progesterone. The glutaraldehyde-fixed samples were post-fixed in OsO_4, dehydrated and embedded in Agar 100^R plastic resin (Agar Aids, Essex, England). Semi-thin sections (1 μm) were cut and stained with buffered toluidine blue for light microscopy. Ultra-thin sections were cut from selected areas with a diamond knife on an ultramicrotome (Ultratome^R, LKB, Sweden), counterstained with uranyl acetate and lead citrate and examined in a Philips 201 EM electron microscope at 60 kV.

Blood sampling and hormone determinations

Blood samples were collected by jugular venipuncture and the plasma separated and stored at –20°C until assayed. Samples were collected daily from the day of last ovulation prior to surgery (both experimental rounds). Once the first signs of prooestrus were noticed, blood was collected at 2-h intervals from 900 to 1700 h and at 4-h intervals from 1700 to 900 h. The frequent blood sampling was stopped 8 h after oophorectomy, but daily sampling continued for 48 h.

Progesterone concentrations were determined according to *Duchens et al.* (1995a). The intra-/inter-assay coefficients of variation were 10.7/13.0% (2.7 nmol L^{-1}), 3.4/7.8% (18.7 nmol L^{-1}) and 7.0/7.5% (55.4 nmol L^{-1})(detection limit: 0.2 nmol L^{-1}). Luteinizing hormone was measured by radioimmunoassay *(RIA, Forsberg et al.* 1993). The intra-/inter-assay coefficients of variation were 6.7/6.6% (3.1 μg L^{-1}), 3.1/8.7% (5.5 μg L^{-1}) and 4.2/9.4% (10.3 μg L^{-1})(detection limit: 0.5 μg L^{-1}). Oestradiol-17β was determined by RIA *(Sirois & Fortune* 1990), with intra-/inter-assay coefficients of variation of 22.7/13.5% (12.9 pmol L^{-1}), 7.3/10.1% (44.1 pmol L^{-1}) and 9.5/6.0% (84.5 pmol L^{-1})(detection limit: 3.5 pmol L^{-1}). Oestradiol-17β and progesterone in follicular fluid were assayed with an enhanced luminescence immunoassay technique (Amerlite Estradiol-60 Assay, Kodak Clinical Diagnostics Ltd., Amersham, UK) on diluted samples, 1:2000 for oestradiol-17ß and 1:10 for progesterone. Serial dilutions of oestradiol-17ß and progesterone yielded displacement curves parallel to the standard curve. All samples were run in a single assay (intra-assay coefficients of variation were 13.6% (oestradiol-17β) and 7.5% (progesterone)).

Statistical analysis

Data were analyzed by ANOVA for repeated measures (SAS, 1987), considering the main effects of treatment, heifer, day of the oestrous cycle and the interaction between treatment and day. Least square means were compared with paired t-tests. Data on plasma hormone concentrations and follicular growth were normalized to the occurrence of luteolysis. The first time when progesterone was below 5 nmol L^{-1}, followed by no further increase, was considered time 0.

Results

The description of the results comprise both experimental rounds (prior to first or second oophorectomy), unless otherwise stated.

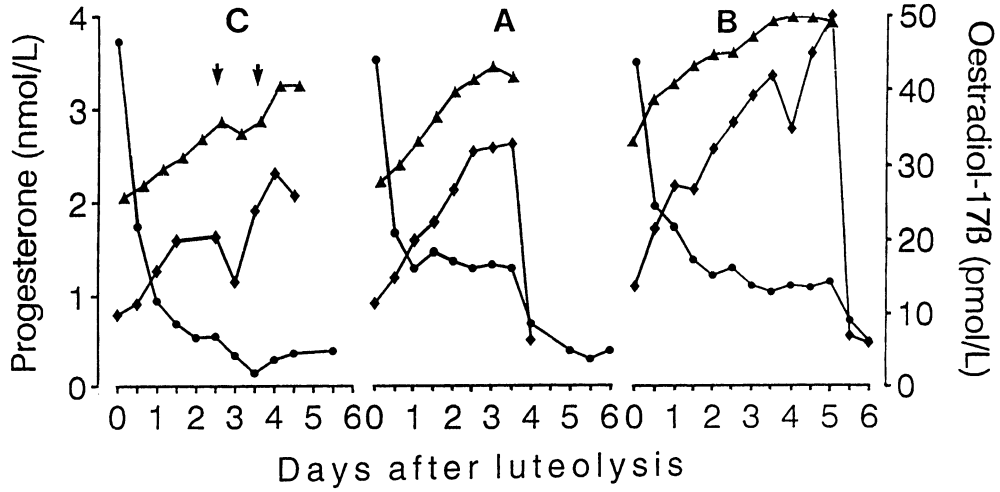

Figure 2, A-C: Least square means of plasma concentrations of progesterone (●), oestradiol-17β (♦), and size of the ovulatory follicle (▲) in heifers during control (Co) and progesterone-treated cycles, in which the ovary was removed (last ▲) at the onset of oestrus (treatment A) or 36 h after the onset of oestrus (treatment B). Arrows in A indicate the occurrence of the peaks of LH.

Oestrous behaviour and endocrine patterns

All heifers, either sham or progesterone implanted, and in both experimental rounds, showed the first symptoms of prooestrus between days 15 to 18, which were followed by oestrous symptoms. Both sham-implanted heifers (control in first and second experimental rounds), one in treatment A and one in treatment B had a clear standing oestrus, whereas those that did not show standing reflex exhibited secondary signs of oestrus as increased uterine tone, a reddening of the vulva and vaginal mucosa and a mucous vulvar discharge. All signs were coincident with the growth of a dominant follicle.

Concentrations of plasma progesterone in sham-implanted heifers (control) decreased abruptly after luteolysis to a basal level of 0.4 nmol L^{-1} (Fig. 2, Co). By contrast, in progesterone implanted heifers (treatment A-B) they were higher ($P<0.05$), reaching 1.3 nmol L^{-1} in the cycles in which the ovary was removed 9 to 12 h after onset of oestrus (Fig. 2, A) and 1.1 nmol L^{-1} in the cycles in which follicular growth was prolonged ($P>0.05$; Fig. 2, B). Plasma oestradiol-17β in the sham-implanted heifers (control) increased during prooestrus, reached a peak at the onset of oestrus and was decreasing at the time of oophorectomy (Fig. 2, Co). In the progesterone-implanted heifers belonging to treatment A, oestradiol-17β levels increased with the same temporal pattern (Fig. 2, A). In the heifers belonging to treatment B, oestradiol-17β also increased but to higher ($P<0.05$) levels (Figure 2, B). The high concentrations were maintained for a longer ($P<0.05$) period, coinciding with the presence of the ovulatory follicle (Fig. 2, B). In all sham- and progesterone implanted heifers, plasma oestradiol-17β decreased rapidly to basal levels once the follicle-bearing ovary had been removed. LH-peaks (12.2 and 12.4 μg L^{-1} of maximum value) were registered at the onset of standing oestrus in the control heifers (Fig. 2, Co). In the

progesterone-treated heifers, no LH-peaks were recorded (Fig. 2, A-B).

The "ovulatory" follicle extended its growth in those heifers belonging to treatment B and reached a maximum size before surgery of 15.8 ± 1.1 mm ($P<0.05$; Fig. 2, B), compared with 13.0 ± 1.5 mm in the sham-control heifers and 13.5 ± 1.1 mm in treatment A-heifers ($P>0.05$; Fig. 2, Co-A). Mean concentrations of oestradiol-17β/progesterone in follicular fluid were 1,200/300 nmol L^{-1} in control animals (Co), 2,600/145 nmol L^{-1} in treatment A, and 5,800/405 nmol L^{-1} in treatment B-heifers. Oestradiol/progesterone ratios were 4 (Co), 18 (A) and 14 (B), respectively.

Ultrastructure of the follicle wall

Sham-implanted (Control) heifers: The round cells of the membrana granulosa appeared to be rather separated from each other in the internal part of the follicle and were connected by numerous gap junctions with longitudinal and annular cross sections *(Maculae & Zonulae,* Fig. 3a). The basal granulosa consisted of a tighter epithelium lying on a well-developed basement lamina (Fig. 3b). Their centrally located nuclei had radial heterochromatin and nucleoli with conspicuous nucleolonemae (Figs. 3a-b). The rough endoplasmic reticulum was dominant consisting mostly of round-to-elongated vesicles (Fig. 3a), but also of concentrically disposed membranes. The mitochondria were predominantly round to elongated, with parallel cristae (Figs. 3a-b). Tubular cristae were less common. Lysosome-like structures and lipid droplets were also frequently observed (Fig. 3a).

The theca interna contained many non-phenestrated capillaries surrounded by a basement membrane. Most of the thecal cells appeared epithelioid in shape, connected by numerous gap junctions (Figs. 3c-d). The nuclei of the epithelioid cells presented heterochromatin located towards the nuclear envelope (Figs. 3c-d). Conspicuous nucleoli, with well-defined nucleolonemae, were also present. Most mitochondria were oval to elongated, with tubular cristae (Figs. 3c-d). The cytoplasm of the thecal cells consisted mostly of agranular endoplasmic reticulum and lipid droplets (Figs. 3c-d).

Progesterone-implanted, treatment A: The ultrastructure of the granulosa and theca interna appeared similar to that described above for the control animals (data not shown).

Progesterone-implanted, treatment B: Two follicles ruptured during surgery. However, the morphology of the follicle wall was similar in all specimens. The granulosa cells appeared to be more dispersed in this group than in the controls, although gap junction cell-to-cell contacts were conspicuous (Fig. 4b). The overall morphology seemed to be similar to that of the control animals, but mild degenerative changes, in the form of large translucent vesicles (intracytoplasmic spaces) and cell debris in the intercellular spaces, were present (Fig. 4a). Large amounts of secondary lysosome-like structures, as well as lipid bodies (Figs. 4a-b) and cells differing in electron density (electron-lucent, Figs. 4b-c), were seen. These cells had less numerous organelles and rather large, intracytoplasmic spaces and empty vesicles (Figs. 4b-c). Cells with concentric, enlarged endoplasmic reticulum profiles were also seen more frequently than in the control specimens (Figs. 4a, 4c).

The organization of the theca interna cells was similar to that in the controls, with most of the cells having an epithelioid appearance (Figs. 5a-b) and features typical of a preovulatory follicle. However, signs of cellular degeneration were more often seen (Figs. 5a-b), both in the form of apoptotic nuclear changes, dilated vesicles in the ergastoplasm or the presence of cells with an increased electron density. Cell

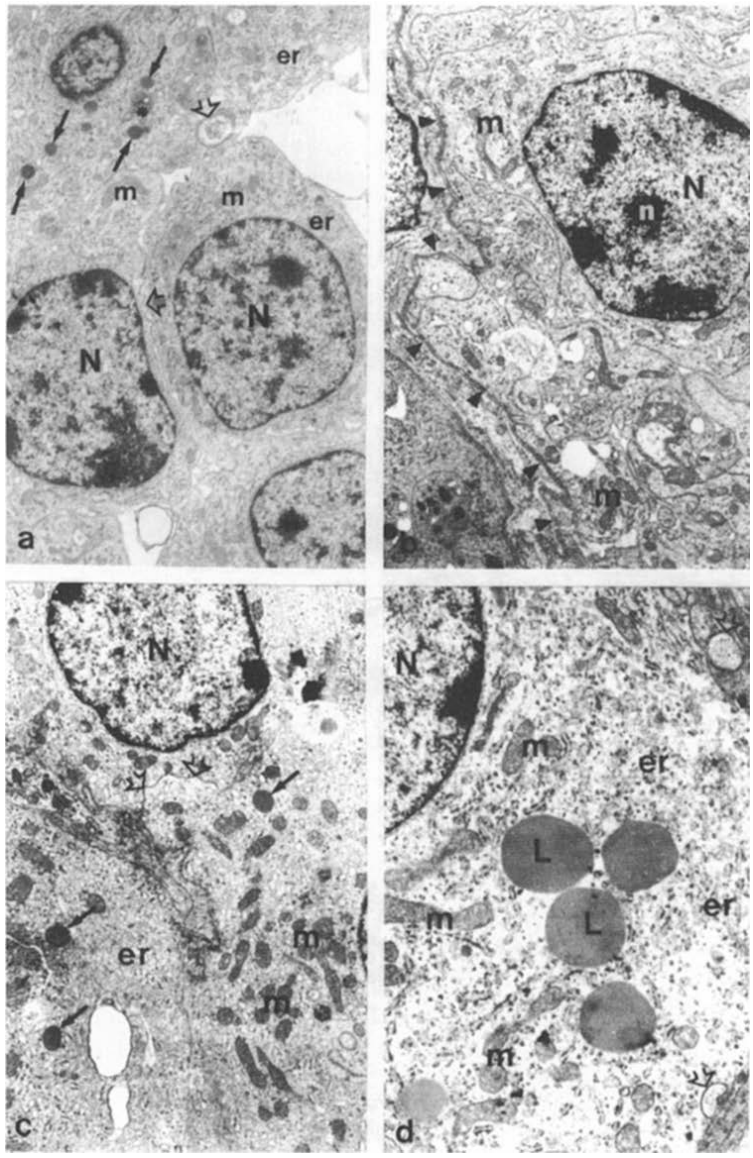

Figure 3 a-d: Transmission electron micrographs of granulosa (a-b, × 5400) and theca-interna (c-d) cells from bovine preovulatory follicles collected 9 to 12 h after the onset of standing oestrus (control cycles, Co). In a, the internal granulosa cells showed centrally-located nuclei (N), and were connected by gap junctions (open arrows), m: mitochondria, er: endoplasmic reticulum, *: lysosome-like vesicles, arrows: lipid droplets. The peripheral granulosa cells (b) were apposed on a basement lamina (arrowheads), m: mitochondria, N: nucleus, n: nucleolus. In c (× 5400) and d (× 7800) numerous gap junctions (open arrows) are seen between theca interna cells, as well as mitochondria (m) with tubular cristae and lipid droplets (arrows in c, L), N: nucleus, er: endoplasmic reticulum.

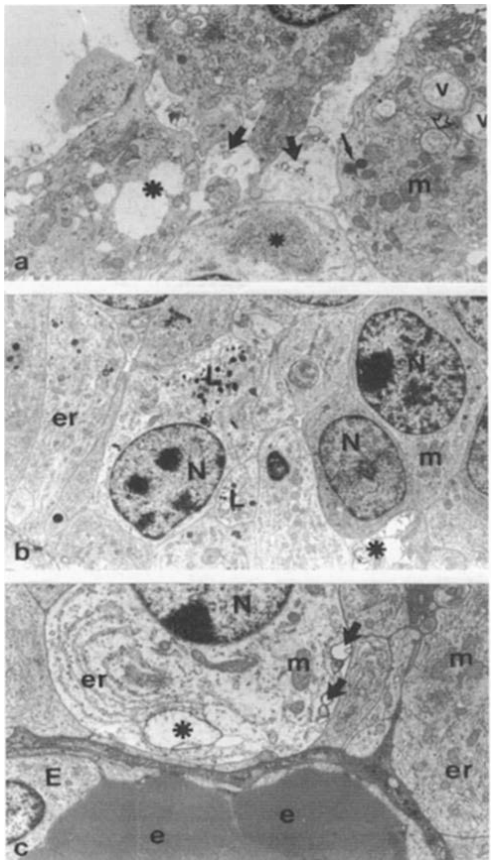

Figure 4 a-c: Transmission electron micrographs of granulosa cells from bovine dominant follicles collected 36 h after the onset of standing oestrus (progesterone-treated cycles, B). In a (×5600), the internal epithelial granulosa cells show marked vesicles (v) and intracytoplasmic spaces (*), and intercellular debris (large arrows), star: concentric-layered er-profiles, m: mitochondria, open arrows: circular gap junctions, small arrow: lipid droplet. In b (×3600) a deeper area of the granulosa layer, showing cells with different cytoplasmic electron-density, can be seen; note the accumulations of lipid droplets (L), *: intracytoplasmic spaces, N: nucleus, m: mitochondria, er: endoplasmic reticulum. In c (×5400) the basal area of the granulosa cell layer is depicted, apposed on the basement membrane and close to a thecal capillary (E: endothelial cell, e: erythrocytes, m: mitochondria, er: endoplasmic reticulum, large arrows: vesicles N: nucleus, *: large vesicle).

debris and myelin figures were also commonly seen (Fig. 5a). No signs of luteinization were present.

Two oocytes were recovered in the sham-implanted animals and one in progesterone-implanted, treatment B. All had a morphology typical of oocytes in the germinal vesicle stage presenting peripherally-located and intact nuclei.

Discussion

The present study showed that suprabasal concentrations of progesterone, induced by subcutaneous implants, allowed the expression of oestrus but extended the growth of the ovulatory follicle beyond the expected time of ovulation. This asynchrony resulted in an altered endocrine pattern, with a greater secretion of oestradiol-17β from the arrested follicle, which was associated with mild morphological alterations in the wall of the ovulatory follicle, but no signs of luteinization. Too few oocytes were recovered to allow any general conclusions to be made. Nevertheless, it is noteworthy that no deviations from normal morphology were observed. Oocytes are usually difficult to retrieve from large follicles. *Maurer & Echternkamp* (1985) reported a higher rate of non-recovered oocytes and embryos in repeat-breeding cows than in normal cattle and suggested that the oocyte degenerated before ovulation. However, another explanation could be that either the oocytes were not released from the ripe follicle or that they ovulated, having an asynchronous nuclear and cytoplasmic maturation due to a resumed meiosis in an abnormal hormonal environment, caused by follicle persistence *(Mihm et al.* 1994).

The endocrine, ovarian and behavioural changes induced by the progesterone-implant treatment were similar to those observed in our previous studies *(Duchens et al.* 1994, *Duchens et al.* 1995a,b). Some treated heifers showed

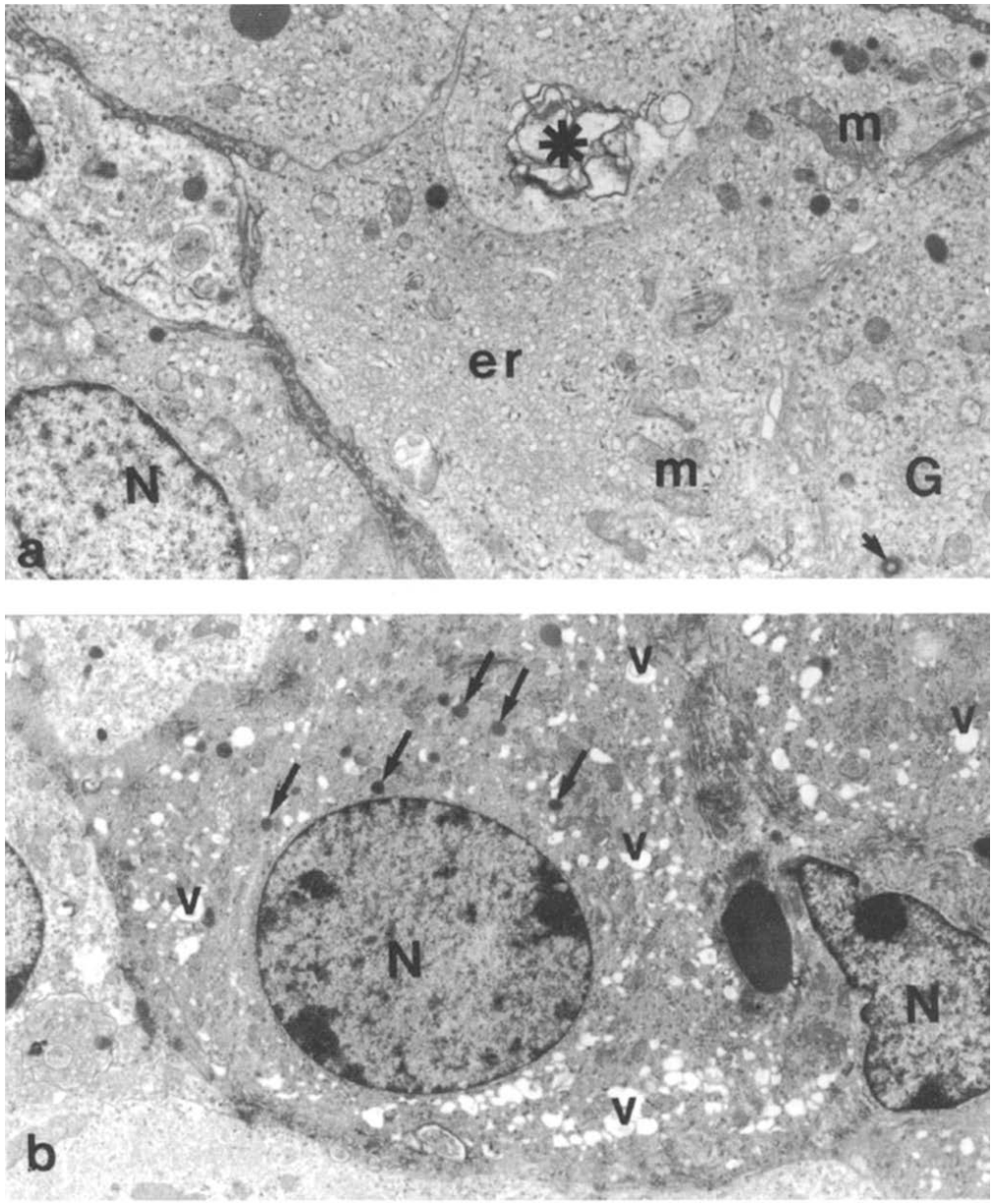

Figure 5 a-b: Transmission electron micrographs of theca-interna cells from bovine dominant follicles collected 36 h after the onset of standing oestrus (progesterone-treated cycles, B), showing myelin figures [* in a (×3600), m: mitochondria, er: endoplasmic reticulum, G: golgi apparatus, small arrow: centriole, N: nucleus] and increased electron-density of the cytoplasm. [b (×2700) note the dilated vesicles (v), arrows: lipid droplets, N: nucleus].

standing oestrus or intense secondary signs of oestrus but, due to the effect of the suprabasal concentration of progesterone, the LH peak was absent and, consequently, the final stages of follicular maturation were arrested. The higher concentrations of oestradiol in cycles in which growth of the ovulatory follicle was prolonged coincided with higher concentrations in the follicular fluid. The oestradiol/progesterone ratio in follicular fluid confirmed that the expected metabolic shift from oestradiol to progesterone production by the preovulatory follicle did not occur, and indirectly showed the absence of a LH surge. Follicular oestradiol concentrations were higher in cycles where oophorectomy was performed 9 to 12 h after standing oestrus than in the control cycles. Although the follicles were collected at the same time after luteolysis and the onset of oestrus, the LH peak was absent in the treated cycles, whereas in the control group the LH peak occurred about 7 h before oophorectomy. This finding corresponds with the results of previous studies showing that the oestradiol concentration in preovulatory follicles reaches its maximum after the onset of oestrus and before the LH surge *(Dieleman et al.* 1983). The LH surge induces a rapid decrease in the oestradiol secretion of the dominant follicle both in vivo *(Dieleman et al.* 1983, *Callesen et al.* 1986) and in vitro *(Staigmiller et al.* 1982).

The morphology of the follicular wall in control cycles (Co) was very similar to that of follicles in cycles with progesterone implants (treatment A), sampled at the same time as controls, and corresponds with what has been described in follicles in early or mid-oestrus in cattle *(Priedkalns & Weber* 1968, *Dieleman et al.* 1983) and sheep *(O'Shea et al.* 1978, *Cran et al.* 1979). The separation of the cells of the membrane granulosa, cumulus and theca interna is indicative of mid-oestrus *(Priedkalns & Weber* 1968, *Cran et al.* 1979), as well as the presence of elongated mitochondria that become rounded in late oestrus *(Priedkalns & Weber* 1968). Morphological and functional luteinization of the membrana granulosa and theca does not become apparent until about 20 h after the LH peak *(Dieleman et al.* 1983). In control cycles, the follicle was removed soon after the occurrence of the LH peak, which explains why no signs of luteinization were observed.

The follicle wall in treatment B cycles did not show any conspicuous abnormalities in connection with the prolonged growth and increased oestradiol secretion of the dominant follicle. The most pronounced change was the dispersion of granulosa cells, indicative of a more advanced follicle *(Priedkalns & Weber* 1968), and mild degenerative changes, such as an accumulation of lysosome-like vesicles, residual bodies and lipid droplets on follicle wall cell components. It remains to be determined whether the lower fertility observed in cases of delayed ovulation *(Bostedt* 1976, *Erb et al.* 1976, *Albihn* 1991) was due to the short-term extended growth of the ovulatory follicle or to persistence of the follicle beyond the time of normal ovulation *(Savio et al.* 1993, *Wehrman et al.* 1993, *Duchens et al.* 1995b). However, changes induced in the oviductal/uterine environment due to the prolonged release of oestradiol by the delayed ovulatory follicle may also contribute to the low fertility. In rats, in which ovulation had been delayed with a subsequent increase in the plasma concentration of oestradiol, *Butcher & Pope* (1979) observed a decrease in implantation rate and retarded embryonic development and, consequently, suggested that oestradiol had a detrimental effect on the maternal milieu. These aspects are presently studied in our laboratory.

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Sammanfattning

Ultrastrukturella förändringar i äggstocksfolliklar hos nötkreatur med experimentell förlängd tillväxtfas orsakad av suprabasala progesteronnivåer

Studien genomfördes för att undersöka om en kort tids förlängning (12-18 timmar efter förväntad ovulation) av tillväxtfasen hos den ovulatoriska follikeln hos kviga, kan orsaka ultrastrukturella förändringar i follikelväggen. Den förlängda tillväxtfasen framkallades genom implantat med progesteron. Kvigorna ovarieektomerades 9-12 timmar (kontroller) och 36 timmar (progesteronbehandlade) efter visad brunst och den ovulatoriska follikeln undersöktes med elektronmikroskopi. Det visade sig att en progesteronkoncentration på cirka 1.2 nmol L^{-1} i blodet gjorde att djuren visade brunst vid förväntad tidpunkt men ovulationen uteblev (ingen LH topp kunde registreras) och follikeln fortsatte att växa. Den förlängda tillväxten medförde att man elektronmikroskopiskt kunde se milda degenerativa förändringar i follikelväggen jämfört med hos kontrolldjuren. Såväl granulosa- som thecaceller hade en ökad elektrontäthet. Antalet sekundära lysosomer och fettdroppar ökade intracellulärt och mellan cellerna ökade mängden celldebris. Inga tecken på luteinisering av follikelväggen kunde skönjas.

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