Effect of GnRH antagonist-induced prolonged follicular phase on follicular atresia and oocyte developmental competence in vitro in superovulated heifers

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A GnRH antagonist (Antarelix) was used to suppress endogenous pulsatile secretion of LH and delay the preovulatory LH surge in superovulated heifers to study the effect of a prolonged follicular phase on both follicle and oocyte quality. Oestrous cycles were synchronized in 12 heifers with progestagen (norgestomet) implants for 10 days. On day 4 (day 0 = day of oestrus), heifers were stimulated with 24 mg pFSH for 4 days and luteolysis was induced at day 6 with PGF2α (2 ml Estrumate). Animals in the control group (n = 4) were killed 24 h after the last FSH injection. At this time, heifers in group A36h (n = 4) and group A60h (n = 4) were treated with 1.6 mg of Antarelix every 12 h for 36 and 60 h, respectively, and then killed. After dissection of ovarian follicles, oocytes were collected for individual in vitro maturation, fertilization and culture; follicular fluid was collected for determination of steroid concentrations, and granulosa cells were smeared, fixed and stained for evaluation of pycnosis rates. Granulosa cell smears showed that 90% of follicles were healthy in the control group. In contrast, 36 and 58% of the follicles in group A36h showed signs of early or advanced atresia, respectively, while 90% of the follicles in group A60h showed signs of late atresia. Intrafollicular concentrations of oestradiol decreased (P < 0.0001) from healthy follicles (799.14 ± 40.65 ng ml⁻¹) to late atretic follicles (3.96 ± 0.59 ng ml⁻¹). Progesterone concentrations were higher (P < 0.0001) in healthy follicles compared with atretic follicles, irrespective of degree of atresia. Oestradiol:progesterone ratios decreased (P < 0.0001) from healthy (4.58 ± 0.25) to late atretic follicles (0.07 ± 0.009). The intrafollicular concentrations of oestradiol and progesterone were significantly higher (P < 0.0001) in the control than in the treated groups. The oestradiol:progesterone ratio was higher (P < 0.0001) in the control (4.55 ± 0.25) than in the A36h (0.40 ± 0.05) and A60h (0.07 ± 0.009) groups. Unexpectedly, the cleavage rate of fertilized oocytes, blastocyst rate and number of cells per blastocyst were not significantly different among control (85%, 41% and 95 ± 8), A36h (86%, 56% and 93 ± 5) and A60h (88%, 58% and 79 ± 4) groups. In addition, there were no significant differences in the blastocyst rates from oocytes derived from healthy (45%), early atretic (54%), advanced atretic (57%) and late atretic follicles (53%). In conclusion, the maintenance of the preovulatory follicles in superovulated heifers with a GnRH antagonist induced more atresia and a decrease in oestradiol and progesterone concentrations. However, the developmental potential in vitro to day 8 of the oocytes recovered from these atretic follicles was not affected.

Introduction

Many studies have shown that the developmental capacity of bovine oocytes may be affected by the size (Pavlok et al., 1992; Lonergan et al., 1994) and by the quality (Hazeleger et al., 1995; Blondin and Sirard, 1994, 1995) of the follicle from which they originate. Follicular atresia is the most likely destiny of any given follicle, and a high proportion of follicles present on the surface of ovaries are to a greater or lesser extent engaged in the process of atresia (Kruip and Dieleman, 1982). Consequently, it is important to know to what extent atresia influences the acquisition of developmental competence by the oocyte and to determine
whether oocytes from atretic follicles, which would normally have been lost due to degeneration in vivo (Sirard and Blondin, 1996), could be ‘rescued’ during in vitro culture to develop until day 8 after insemination.

The criteria usually used for the determination of atresia include the steroid concentration of the follicular fluid (Grimes and Ireland, 1986), the pattern of insulin-like growth factor binding protein (IGFBP) expression (Monget et al., 1993) and the rate of pycnosis or the extent of apoptosis in granulosa cells (Jolly et al., 1997a,b; Huet et al., 1998). Follicular atresia is characterized by increased progesterone and decreased oestradiol production by the follicle (Ireland and Roche, 1982; Spicer et al., 1987). Conversely, healthy follicles have relatively high oestradiol and low progesterone concentrations. The variations in the concentration of these steroids in follicular fluid are consecutive to changes in steroidogenic enzyme expression and activity in follicular cells, particularly a loss in aromatase and 17α-hydroxylase C17,20-lyase in granulosa and theca cells, respectively (Rodgers et al., 1986; Xu et al., 1995; Huet et al., 1997). These changes in the production rates of steroids by follicular cells may affect the developmental capacity of oocytes. It has been reported that follicles containing oocytes capable of forming blastocysts have lower progesterone concentrations than those containing oocytes that do not develop to the blastocyst stage, a characteristic of non-atretic follicles, whereas oestradiol concentrations are not related to the developmental potential of the oocyte (Hazeleger et al., 1995). In contrast, a positive correlation has been shown between the percentage of blastocysts and the mean oestradiol concentrations of the follicular fluids of the follicles from which the oocytes originated (Van de Leemput et al., 1998). Driancourt et al. (1998) showed that granulosa cells of follicles containing competent oocytes (measured as the ability to develop to the blastocyst stage) had an increased aromatase activity compared with cells of follicles containing incompetent oocytes, although the steroid content of the follicular fluid was not affected.

A clear relationship between follicular atresia and the maintenance of oocyte developmental competence has not yet been fully established. The degree of atresia cannot be measured easily for each follicle from which an oocyte is harvested unless dissection is used on all of them (Blondin and Sirard, 1995). The parallel study of atresia and oocyte competence requires the use of in vitro embryo production methods designed for individual culture (Carolan et al., 1996). In addition, atresia does not occur synchronously in follicles under physiological conditions and the kinetics of functional changes in follicular cells and oocytes during atresia are difficult to assess.

In the present study, the GnRH antagonist model has been used to suppress LH pulses and delay the preovulatory LH surge (Rieger et al., 1989) in superovulated heifers to induce atresia in potentially preovulatory follicles and to determine the effect of degree of atresia and intrafollicular concentrations of steroids on oocyte developmental competence, as assessed by their individual maturation, fertilization and development in vitro.

**Materials and Methods**

**Materials**

The following materials were used: bovine serum albumin (BSA; Sigma fraction V Sigma, St Louis, MO); tissue culture medium 199 (TCM 199; Sigma); fetal calf serum (FCS; Sigma); mineral oil (Sigma); heparin-sodium salt (167 iu mg–1; Calbiochem, San Diego, CA); Percoll (Pharmacia, Uppsala); synthetic oviduct fluid (Takahashi and First 1992, containing 3 mg BSA ml–1); essential amino acid (Sigma MEM) and non-essential amino acid (Sigma MEM); epidermal growth factor (EGF; Sigma); semen (CIA, URCO; Rouillon). pFSH (LH contamination < 0.7% determined by radioreceptor and radioimmunoassay; J. F. Beckers, Liège).

**Animals and treatment**

Oestrous cycles were synchronized in 12, two-year-old, cyclic Charolais heifers with a 10 day Norgestomet implant. Each heifer received a decreasing-dose regimen twice a day of i.m. injections of purified pig FSH (pFSH) for 4 days (5, 5, 4, 4, 2, 2, 1 and 1 mg per injection; total dose = 24 mg). The treatment was initiated on day 4 of the synchronized cycle (day 0 = day of oestrus). Luteolysis was induced at day 6 with 15 mg PGF2α (2 ml Estrumate) i.m. Animals in the control group (group C; n = 4) were killed 24 h after the end of pFSH treatment. At this time, heifers in groups A36h (n = 4) and A60h (n = 4) were treated with 1.6 mg GnRH antagonist (Antarelix, Europeptides, Argenteuil) at a dose of 1.6 mg every 12 h (Madill et al., 1994) for 36 and 60 h, respectively, and then killed.

Jugular blood samples were taken when animals were killed in the control group and before Antarelix injection to detect plasma LH surge and, at 36 and 60 h in the treated groups, to detect decreased plasma LH concentrations.

**Follicular fluid and classification**

After the animals were killed and the ovaries were recovered, a total of 170 individual follicles (diameter > 7 mm) were dissected free of ovarian tissue. The number of follicles per group were: n = 64 (group A36h), n = 52 (group A60h) and n = 54 (control group). Follicular fluid from each follicle was collected, centrifuged at 300 g for 5 min, and the supernatant was stored at –20°C until steroid concentrations were determined by radioimmunoassay.

Follicular quality was determined as described by Monniaux (1987). Briefly, for each follicle, a smear of granulosa cells was prepared on histological slides, fixed in ethanol-formaldehyde-acetic acid (80:15:5) and stained with Feulgen. Twenty fields were observed for each smear of granulosa to quantify the frequency of pycnotic bodies. Follicles were classified as healthy (rare or no pycnotic granulosa cells), early atretic (< 20 pycnotic bodies), advanced atretic (> 20 pycnotic bodies) or late atretic (numerous pycnotic bodies > 20) in each field in smear of granulosa cells.
In vitro maturation, fertilization and culture

The methods used for in vitro maturation, fertilization and culture were those described by Carolan et al. (1996). Briefly, after follicular dissection, oocytes were recovered and washed four times in modified phosphate buffered saline (PBS), supplemented with 0.3 mmol pyruvate l–1, 50 µg gentamycin ml–1 and 0.5 mg BSA ml–1. Oocytes were then rinsed in maturation medium and cultured individually for 24 h in 10 µl droplets at 39°C in an atmosphere of 5% CO2 in air with maximum humidity. The maturation medium used was TCM 199 supplemented with 10 ng EGF ml–1 (Lonergan et al., 1996).

After maturation, oocytes were again washed in PBS and then rinsed in fertilization medium (TALP; Parrish et al., 1986), supplemented with 10 µg heparin ml–1 and transferred into 25 µl oil overlayed droplets of the same medium. A sample of motile spermatozoa was obtained using frozen-thawed semen overlaid onto a Percoll discontinuous density gradient (2 ml at 45% over 2 ml at 90%) and centrifuged for 20 min at 700 g at room temperature. The supernatant was aspirated and the remaining spermatozoa resuspended in TALP and washed by centrifugation at 100 g for 10 min. The same ejaculate from one bull was used for all experiments. Spermatozoa were counted in a haemocytometer and diluted in fertilization medium to a concentration of 4·106 spermatozoa ml–1; this sperm suspension was added (25 µl) to each fertilization droplet to obtain a final concentration of 2·106 spermatozoa ml–1. Gametes were co-incubated for 18–21 h in 5% CO2 in humidified air at 39°C. Presumptive zygotes were denuded of surrounding cumulus cells 18 to 21 h after insemination by repeated pipetting and subsequent washing in PBS and synthetic oviduct fluid and were then transferred to the culture droplets (10 µl) overlaid with mineral oil and cultured at 39°C in an atmosphere of 5% CO2, 5% O2, 90% N2. Fetal calf serum was added (10% (v/v) heat treated) to the culture drops 24 h later (that is, 48 h after insemination). The medium was not changed throughout the 8 day culture period. The cleavage rate and percentage of embryos at the five- to eight-cell stage were recorded on day 2 of culture (72 h after insemination). The number of embryos developing to the blastocyst stage was assessed on days 6–8 of culture. Day 8 blastocysts from treated and control groups were fixed in ethanol (100%), air dried and stained with Hoechst 33342 (10 µg ml–1 in 2.3% (v/v) sodium citrate solution). The slides were observed under an epifluorescence microscope (filter 470 nm), and the total number of cells was counted.

Radioimmunoassay

Follicular fluids were assayed for oestradiol, progesterone and androstenedione. The methods used for follicular steroid assay were adapted from assays of plasma samples with extraction. A 25 µl aliquot of follicular fluid was diluted with 1 ml distilled water and extracted with 10 ml ethyl acetate–cyclohexane (1:1, v/v). Each sample was measured in duplicate.

The antiserum for oestradiol (Saumande, 1981) crossreacted with 6-ketoestradiol (12.5%) 16-epiestriol (5%), 16-ketoestradiol (1.3%) and < 1% with other steroids. The limit of detection of the assay was 1 pg per tube and the intra-assay coefficients of variation were 8.6% and 9.4%, respectively, for the doses of 2 and 25 ng ml–1.

The antiserum for progesterone (Saumande et al., 1985) crossreacted slightly with 20-hydroxyprogesterone (1.7%) and < 1% with other steroids. The limit of detection of the assay was 12 pg per tube and the intra-assay coefficients of variation were 18.0 and 15.0%, respectively, for the doses of 16 and 0.5 ng ml–1.

The antiserum for androstenedione (Williams and de Reviers, 1981) crossreacted with testosterone (7%), 0.2% with dehydroepiandrosterone and < 0.1% with other steroids. The limit of detection of the assay was 3 pg per tube and intra-assay coefficients of variation were 11.6% and 15%, respectively, for the doses of 10 and 0.5 ng ml–1.

The volume of plasma assayed in duplicate was 50 µl for LH (Pelletier et al., 1982). The potency of the standards used relative to NIH was 1.8 × NIH-IHS1; the limit of detection of the assay was 15 pg per tube. The intra-assay coefficient of variation was 8%.

Statistical analysis

Cleavage rate, percentage of five- to eight-cell embryos and blastocyst yield were compared by chi-squared analysis. The number of cells in blastocysts and the steroid concentrations were analysed by Student’s t test. Steroid concentrations, the oestradiol:progesterone and androstenedione:oestradiol ratios related to developmental stage were compared using the Mann–Whitney U test.

Results

LH concentration

No LH surge was observed before control animals were killed or before GnRH antagonist administration (Antarelix) in treated groups. Plasma LH concentrations decreased (P < 0.01) from 0.6 ± 0.03 ng ml–1 to 0.3 ± 0.04 and 0.3 ± 0.02 ng ml–1 at 36 h and 60 h, respectively, in the treated groups. The LH concentration was 0.7 ± 0.2 ng ml–1 in the control group.

Follicle distribution

In the control group, analysis of the smears of granulosa cells from individual follicles demonstrated that 91% of follicles were healthy and that the remaining 9% were early atretic. In contrast, the majority of follicles (90%) in the A60h group were late atretic with 10% in advanced atresia while, in the A36h group, 36, 58 and 6% of follicles were in early, advanced and late atretic. In contrast, the majority of follicles (90%) in the A60h group were late atretic with 10% in advanced atresia while, in the A36h group, 36, 58 and 6% of follicles were in early, advanced and late atresia, respectively.

Intrafollicular concentrations of steroids

The oestradiol, progesterone and androstenedione concentrations are presented (Table 1). The oestradiol:
progesterone ratios decreased significantly ($P < 0.0001$) from healthy (4.58 ± 0.25) to early atretic (1.33 ± 0.37), advanced atretic (0.29 ± 0.04) and late atretic (0.07 ± 0.009) follicles. The androstenedione:oestradiol ratios increased significantly ($P < 0.0001$) from healthy (0.05 ± 0.009) to early atretic (1.29 ± 0.27), advanced atretic (4.75 ± 1.94) and late atretic (29.65 ± 8.92) follicles.

**Embryo development after in vitro maturation, fertilization and culture**

There was no significant difference in the cleavage rate at 72 h after insemination between the groups (Table 2). However, the number of embryos at the five- to eight-cell stage 72 h after insemination was significantly higher ($P < 0.05$) in follicles giving rise to oocytes (non-cleaved, 2–16 cells, > 16 cells and blastocysts) than in those giving rise to oocytes developing to the 2–16 cell, > 16 cell or blastocyst stages. In the control, A36h and A60h groups, the intrafollicular concentrations of oestradiol were significantly higher in the control than in the treated groups (A36h or A60h). In addition, the intrafollicular concentration of oestradiol was significantly higher ($P < 0.0001$) in the control than in the A36h and A60h groups, the intrafollicular concentrations of oestradiol were significantly higher in the control than in the treated groups (A36h or A60h).

Oestradiol concentrations and oestradiol:progesterone ratios for healthy follicles, early, advanced and late atretic follicles in relation to the developmental stage attained by the embryos are presented (Table 3).

**Relationship between intrafollicular concentrations of steroids and developmental stage of embryos**

**Oestradiol.** Irrespective of the developmental stage attained by the embryos (non-cleaved, 2–16 cells, > 16 cells and blastocysts), the intrafollicular concentration of oestradiol was higher ($P < 0.0001$) in the control than in the treated groups (A36h or A60h). In addition, the intrafollicular concentration of oestradiol was significantly higher ($P < 0.0003$) in the A36h than in the A60h group (Fig. 1). In the control, A36h and A60h groups, the intrafollicular concentrations of oestradiol were not significantly different when related to the stage of embryo development. The oestradiol:progesterone ratio was higher ($P < 0.0001$) in the control (4.55 ± 0.25) than in the A36h (0.40 ± 0.05) and A60h (0.07 ± 0.009) groups.

**Progesterone.** Irrespective of the developmental stage attained by the embryos (non-cleaved, 2–16 cells, > 16 cells and blastocysts), the intrafollicular concentrations of progesterone were significantly higher in the control than in the treated-groups (A36h or A60h) ($P < 0.0001$) and no significant difference was observed between the A36h and A60h groups (Fig. 2). In the control group, progesterone concentrations were lower ($P < 0.01$) in follicles giving rise to non-cleaved oocytes than in those giving rise to oocytes developing to the 2–16 cell, > 16 cell or blastocyst stages. In the A36h and A60h groups, the intrafollicular concentrations of progesterone were not significantly different when related to the stage of embryo development.

**Table 1.** Oestradiol, androstenedione and progesterone concentrations (ng ml⁻¹ ± SEM) in follicular fluid from bovine follicles classified according to their degree of atresia

<table>
<thead>
<tr>
<th>Steroids (ng ml⁻¹)</th>
<th>Healthy (n = 49)</th>
<th>Early atretic (n = 28)</th>
<th>Advanced atretic (n = 42)</th>
<th>Late atretic (n = 51)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oestradiol</td>
<td>799.14 ± 40.65a</td>
<td>139.92 ± 54.38b</td>
<td>14.01 ± 1.33c</td>
<td>3.96 ± 0.59d</td>
<td>$P &lt; 0.0001$</td>
</tr>
<tr>
<td>Progesterone</td>
<td>195.12 ± 9.28a</td>
<td>63.99 ± 8.82a</td>
<td>58.41 ± 5.13b</td>
<td>54.05 ± 3.51b</td>
<td>$P &lt; 0.00001$</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>41.53 ± 4.98a</td>
<td>33.51 ± 7.14b</td>
<td>21.40 ± 2.91b</td>
<td>33.57 ± 4.31ab</td>
<td>$P &lt; 0.001$</td>
</tr>
</tbody>
</table>

The degree of atresia was assessed from smears of granulosa cells. $n$, number of follicles. Different letters indicate significant differences among means within each row of the table.

**Table 2.** Development of bovine oocytes after individual in vitro maturation, fertilization and culture

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$n$</th>
<th>Cleavage at 72 h after insemination</th>
<th>Development 8 days after insemination</th>
<th>Number of cells (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total (%)</td>
<td>Five- to eight-cell embryos (%)</td>
<td>Morulae/blastocysts (%)</td>
</tr>
<tr>
<td>Control</td>
<td>54</td>
<td>46 (85)</td>
<td>32 (59)$^a$</td>
<td>22 (41)$^a$</td>
</tr>
<tr>
<td>36 h</td>
<td>64</td>
<td>55 (86)</td>
<td>50 (78)$^a$</td>
<td>39 (61)$^a$</td>
</tr>
<tr>
<td>60 h</td>
<td>52</td>
<td>46 (88)$^a$</td>
<td>44 (85)$^a$</td>
<td>30 (58)$^a$</td>
</tr>
</tbody>
</table>

Oocytes were recovered from superovulated heifers killed 24 h after the last FSH injection (control) or maintained for an additional 36 or 60 h under Antarelix inhibition of LH surge before the animals were killed.

$n$, number of oocytes. $^a$ versus $^b$ $P < 0.05$, between experimental groups.
Androstenedione. There was no significant difference in the intrafollicular concentrations of androstenedione in the control, A36h and A60h groups in relation to the stage of embryo development.

Discussion

Antarelix treatment for 36 or 60 h after superovulation induced different degrees of follicular atresia (early, advanced and late atresia) as compared with the controls, in which most (91%) follicles were healthy. The use of in vitro

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### Table 3. Development of bovine oocytes after individual in vitro maturation, fertilization and culture, oestradiol concentrations (ng ml⁻¹ ± SEM) and oestradiol:progesterone ratio (± SEM)

<table>
<thead>
<tr>
<th>Development</th>
<th>Healthy (n = 49)</th>
<th>Early atretic (n = 28)</th>
<th>Advanced atretic (n = 42)</th>
<th>Late atretic (n = 51)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oestradiol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>788.37 ± 110.2 a</td>
<td>605.77 ± 251.27 b</td>
<td>16.50 ± 2.14 c</td>
<td>2.24 ± 0.43 d</td>
<td></td>
</tr>
<tr>
<td>Oestradiol:progesterone</td>
<td>5.23 ± 0.410 a</td>
<td>4.49 ± 1.877 b</td>
<td>0.29 ± 0.03 e</td>
<td>0.041 ± 0.008 f</td>
</tr>
<tr>
<td>2–16 cells n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16 (33) a</td>
<td>7 (25) b</td>
<td>4 (10) b</td>
<td>14 (27) a</td>
<td></td>
</tr>
<tr>
<td>Oestradiol</td>
<td>744.36 ± 59.11 a</td>
<td>238.67 ± 145.83 c</td>
<td>16.00 ± 3.43 b</td>
<td>3.80 ± 0.85 c</td>
</tr>
<tr>
<td>Oestradiol:progesterone</td>
<td>4.04 ± 0.49 a</td>
<td>1.52 ± 0.77 b</td>
<td>0.93 ± 0.33 c</td>
<td>0.077 ± 0.01 b</td>
</tr>
<tr>
<td>&gt; 16 cells* n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26 (53)</td>
<td>18 (64)</td>
<td>31 (74)</td>
<td>29 (57)</td>
<td></td>
</tr>
<tr>
<td>Oestradiol</td>
<td>836.86 ± 61.4 a</td>
<td>23.87 ± 2.24 b</td>
<td>12.68 ± 1.59 c</td>
<td>4.52 ± 0.94 d</td>
</tr>
<tr>
<td>Oestradiol:progesterone</td>
<td>4.59 ± 0.36 a</td>
<td>0.64 ± 0.12 b</td>
<td>0.23 ± 0.03 c</td>
<td>0.08 ± 0.01 d</td>
</tr>
<tr>
<td>Blastocyst n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22 (45)</td>
<td>15 (54)</td>
<td>24 (57)</td>
<td>27 (53)</td>
<td></td>
</tr>
<tr>
<td>Oestradiol</td>
<td>823.24 ± 69.19 a</td>
<td>24.11 ± 2.60 b</td>
<td>10.24 ± 1.30 c</td>
<td>4.67 ± 1.01 d</td>
</tr>
<tr>
<td>Oestradiol:progesterone</td>
<td>4.43 ± 0.39 a</td>
<td>0.67 ± 0.14 b</td>
<td>0.19 ± 0.03 c</td>
<td>0.08 ± 0.01 d</td>
</tr>
</tbody>
</table>

Oocytes were recovered from follicles of different status (healthy, early atretic, advanced atretic and late atretic) in superovulated heifers killed 24 h after the last FSH injection in the control, or maintained for an additional 36 or 60 h under Antarelix inhibition of the LH surge before the animals were killed.

*Including blastocysts.

Different letters indicate differences among means within each row of the table. abc dP < 0.01.
embryo production techniques designed for individual culture permitted the evaluation of the relationship between the ability of the oocyte to develop to the blastocyst stage and the characteristics of the follicle from which it originated.

The androgen:oestradiol ratio increases in the follicular fluid of ovine atretic follicles (Carson et al., 1981) and the concentrations of progesterone in the fluid of healthy, medium-sized bovine follicles are lower than in atretic and luteinized-atretic follicles (Spicer et al., 1987). Similarly, high progesterone concentrations have been reported in large atretic follicles (Ireland and Roche, 1982; Kuyp and Dieleman 1985). In contrast, progesterone concentrations in follicular fluid remain unchanged during atresia of large follicles in sheep (Moor et al., 1978) and humans (Bomsel-Helmreich et al., 1979; McNatty, 1981) and are decreased during atresia of large follicles in mares (Condon et al., 1979). In the present study, intrafollicular oestradiol concentrations decreased with the degree of atresia, while intrafollicular progesterone concentrations were higher in healthy follicles than they were in atretic follicles. The lower intrafollicular concentrations of progesterone observed in atretic follicles originating from heifers treated with a GnRH antagonist may be the consequence of reduced ovarian steroidogenesis (Campbell et al., 1990; Oussaid et al., 1999) and the absence of luteinization caused by lower plasma LH concentrations after Antarelix treatment. The oestradiol:progesterone ratio was higher in healthy than it was in atretic follicles and decreased significantly with the degree of atresia. In the present experimental model, the decreased oestradiol:progesterone ratio resulted from the reduced intrafollicular concentrations of oestradiol from healthy to late atretic follicles rather than by an increase of progesterone concentrations with increasing degree of follicular atresia.

Previous reports showed that large follicles contain oocytes more capable than smaller follicles of developing to the blastocyst stage (Pavlak et al., 1992; Lonergan et al., 1994), and that follicular atresia in its early stages can, in fact, promote acquisition of developmental competence (Blondin and Sirard, 1995). This finding is not so surprising when it is considered that certain events occurring during the early stages of atresia are similar to those occurring after the LH surge in the preovulatory follicle (Wise and Maurer, 1994; Wise et al., 1994). In the present study, only large, potentially ovulatory, follicles of superovulated heifers were dissected. The blastocyst rate at day 8 after insemination was not different between the control group and the groups treated with Antarelix for 36 or 60 h. The difference observed in the proportion of five- to eight-cell stage embryos at 72 h after insemination between the control and treated groups may be due to differences in developmental kinetics. Oocytes originating from early, advanced and late atretic follicles exhibited cleavage and blastocyst rates similar to those of oocytes derived from healthy follicles. Irrespective of the degree of follicular atresia, oocyte developmental competence in vitro was not affected in large preovulatory follicles prolonged with GnRH antagonist in superovulated heifers, despite a decrease in intrafollicular concentrations of oestradiol and a significant decrease in the oestradiol:progesterone ratio. However, maintaining the follicle in prolonged dominance by prostegins in cattle (Sirois and Fortune, 1990; Savio et al., 1993a; Stock and Fortune, 1993) induces high LH pulse frequency (Roberson et al., 1989; Savio et al., 1993b). This increased LH pulse frequency in prolonged dominant follicles induces the oocyte to undergo premature maturation (resumption of meiosis) in vivo (Mihm et al., 1994; Revah and Butler, 1996) and leads to poor fertility after insemination (Savio et al., 1993a; Ahmed et al., 1995). Premature luteinization in prolonged dominant follicles may also be a factor involved in the decreased fertility of oocytes from these follicles (Bigelow and Fortune, 1998). In the GnRH antagonist model used in the present study, suppressed LH pulsatility overcomes the risks of premature maturation of oocytes and luteinization of follicles. There is also evidence that, during the follicular phase, over-exposure to LH may be detrimental to oocyte quality in cattle (Boland et al., 1991), rats (Mattheij et al., 1994) and rabbits (Bomsel-Helmreich et al., 1989). It can be concluded that a GnRH-induced prolonged follicular phase in superovulated heifers is not detrimental in terms of oocyte quality and is also permissive of follicular atresia.

Many attempts have been made, especially in the field of human IVF, to correlate oocyte quality with the concentration of various components of the follicular fluid from which it originated, including steroids (Morgan et al., 1990; Stubbings et al., 1990; de Sutter et al., 1991; Itskovitz et al., 1991; Tarlatzis et al., 1993; Artini et al., 1994; Suchanek et al., 1994), growth hormone (Tarlatzis et al., 1993), growth factors (Artini et al., 1994) and FSH (Suchanek et al., 1994). In cattle, follicles containing oocytes capable of forming blastocysts had lower progesterone concentrations than did follicles containing oocytes that did not develop to the blastocyst stage (Hazeleger et al., 1995). In addition, developmental potential was not related to the follicular fluid oestradiol concentrations. In contrast, Van de Leemput et al. (1998) reported a positive correlation between the percentage of blastocysts and the mean follicular fluid oestradiol concentration of the donating follicle. The experimental model in the present study demonstrated that intrafollicular concentrations of oestradiol were 5-, 57- and 200-fold higher in healthy follicles than they were in early, advanced and late atretic follicles, respectively. In addition, the intrafollicular concentration of progesterone was threefold higher in healthy follicles than it was in atretic follicles without any effect on blastocyst rates. A correlation was observed between high intrafollicular androgen: oestrogen ratios and the inability of the oocyte to complete meiotic maturation in human antral follicles (McNatty et al., 1979) and in macaques treated with an aromatase inhibitor to increase the endogenous ratio of androgen:oestrogen (Zelinski-Wooten et al., 1993). The results of the present study show an increasing androgen:oestrogen ratio from 0.05 ± 0.009 in healthy follicles to 26.65 ± 8.92 in late atretic follicles without any effect on blastocyst rate.

Oocyte developmental competence seems to be acquired at different stages of follicular growth, since some oocytes originating from large follicles fail to produce embryos, while some oocytes from medium-sized follicles already have this capacity (Sirard and Blondin, 1996). Oocytes that have not acquired this competence during folliculogenesis remain capable of resuming meiosis and being fertilized, but they are unable to develop to the blastocyst stage. The kinetics of the acquisition of oocyte developmental competence during
foliculogenesis, and the oocyte and follicular factors involved remain to be elucidated. The results of the present study demonstrate that oocytes are capable of retaining their developmental competence in vitro despite morphological signs of late follicular atresia in the preovulatory follicle of superovulated heifers treated with a GnRH antagonist.

In conclusion, the maintenance of the preovulatory follicle with a GnRH antagonist treatment in superovulated heifers was detrimental to follicular health, inducing high concentrations of atresia and a decrease in oestradiol and progesterone concentrations in follicular fluids. However, the developmental potential in vitro of the oocytes recovered from these atretic follicles was unaffected, as was the quality of the resulting embryos, as indicated by the blastocyst rate and number of cells. These findings indicate that, once the oocyte has attained its full developmental competence (that is, in the preovulatory follicle of superovulated heifers), suppression of LH pulses renders the oocyte less dependent on the subsequent fate of the follicle.

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