Meiotic competence of in vitro grown goat oocytes

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The objective of the present study was to grow meiotically incompetent goat oocytes from early antral follicles in vitro and to render them competent to undergo germinal vesicle breakdown. Cumulus–oocyte complexes with pieces of parietal granulosa cells were isolated from follicles 0.35–0.45 mm in diameter using both mechanical and enzymatic methods. The cumulus–oocyte complexes were divided into two groups according to oocyte diameter (group A: < 0.5 μm; group B: > 0.5 μm) and cultured for 8 or 9 days on granulosa cell monolayers. Within 8 days of culture, the mean oocyte diameter increased from 86 ± 0.4 μm to 95 ± 0.7 μm in group A and from 106 ± 0.2 μm to 109 ± 0.5 μm in group B. After 9 days of culture, the mean diameter of oocytes from groups A and B were 99 ± 0.5 μm and 112 ± 0.4 μm, respectively. The meiotic competence of oocytes grown in vitro was evaluated by in vitro maturation. Within 8 days of culture, only 3% of oocytes from group A and 6% of oocytes from group B acquired the ability to undergo germinal vesicle breakdown. After 9 days of culture, 7% of group A oocytes and 42% of group B oocytes were competent to resume meiosis. The expression of p34cdc2 in oocytes grown in vitro was analysed by the western blot technique. During 9 days of culture, p34cdc2 accumulated in both groups of growing oocytes, but its concentration was lower than in fully grown oocytes used as controls. The results showed for the first time that goat oocytes from early antral follicles can grow, accumulate p34cdc2 and acquire the ability to resume meiosis, when cultured for 9 days on granulosa cell monolayers.

Introduction

Throughout folliculogenesis, mammalian oocytes are arrested at the diplotene stage of the first meiotic prophase, or germinal vesicle (GV) stage. Meiotic competence is defined as the ability of an oocyte to undergo germinal vesicle breakdown (GVBD), progress to metaphase I (MI), extrude the first polar body and reach metaphase II (MII). Meiotic competence is sequentially acquired during the final phase of oocyte growth. Oocytes initially acquire the ability to undergo GVBD, arresting at about MI. During further development, they acquire the ability to reach MII, and thereby become meiotically competent. Meiotic competence of oocytes is acquired in late preantral follicles in mice (Sorensen and Wassarman, 1976) and in antral follicles between 0.5 and 2–3 mm in diameter in preantral follicles in mice (Sorensen and Wassarman, 1976) and goats (De Smedt et al., 1994). Goat oocytes from early antral follicles (< 0.5 mm in diameter) were classified as incompetent since only 7% of them were able to undergo GVBD when matured in vitro. Oocytes became GVBD-competent in follicles 0.5–0.8 mm in diameter, but were arrested in prometaphase I. During subsequent follicle growth, the oocytes acquired the ability to progress to MI in follicles 1.0–1.8 mm in diameter, and to achieve nuclear maturation up to MII in follicles > 2 mm in diameter.

Reinitiation of meiosis in oocytes is controlled by the maturation promoting factor (MPF) first described in Xenopus by Masui and Markert (1971). MPF is a protein kinase composed of a regulatory subunit, cyclin B, and a catalytic subunit, p34cdc2 (Lohka et al., 1988). The protein kinase activity of MPF is regulated by the association of p34cdc2 with cyclin B and by phosphorylation-dephosphorylation events. In goats, as in other mammalian species (for review, see Eppig, 1996), MPF activity increases at GVBD, reaching a maximum value at MI, and decreasing during the anaphase–telophase transition to increase again to another maximum value at MII (Dedieu et al., 1996). After in vitro maturation (IVM), goat oocytes from follicles < 0.5 mm in diameter remained at the GV stage and did not exhibit MPF activity (Dedieu et al., 1996). These oocytes were deficient in p34cdc2, the catalytic subunit of MPF (Dedieu et al., 1998) whereas they were already equipped with the regulatory subunit, cyclin B (Hue et al., 1997). These results indicate that a deficiency in the expression of p34cdc2 may be one limiting factor for the acquisition of GVBD competence of goat oocytes.

A large number of oocytes are contained in the ovaries, but only meiotically competent oocytes can be used for IVM. Consequently, the number of embryos that can be produced in vitro from a particular donor is limited. Incompetent oocytes from early antral or preantral follicles might also be used in the future for livestock production, provided that...
adequate systems to grow them in vitro are developed. Mouse oocytes co-cultured with somatic cells can acquire GVBD competence in vitro over a period comparable with that experienced in vivo (Eppig, 1977). Furthermore, live young have been produced from mouse oocytes grown, matured and fertilized in vitro (Eppig and Schroeder, 1989). Efficient systems that support the growth of ruminant oocytes in vitro are required to provide a valuable means of increasing the number of embryos produced in vitro from animals of agricultural interest and to enlarge the population of transgenic or endangered animals. However, oocyte growth in ruminants is very slow and this may explain the limited success obtained in these species compared with that in mice. Only 5% of bovine oocytes from early antral follicles (0.5–0.7 mm in diameter), cultured with granulosa cells for 14 days, acquired meiotic competence and were penetrated by spermatozoa after in vitro insemination (Osaki et al., 1997). With the goat model, it would be possible to define conditions in vitro that would make it possible for a given class of oocytes to reach a more advanced stage of development.

In the present study, an attempt was made to grow oocytes from early antral follicles (< 0.5 mm) in vitro and to render them competent to undergo GVBD. The expression of p34^cdc2 was also analysed by western blotting in these oocytes grown in vitro.

**Materials and Methods**

Ovaries were collected from 2-5-year-old adult goats of French Alpine and Saanen breeds. All procedures relating to the care and use of animals were approved by the French Ministry of Agriculture according to the French regulations (instruction 19/04/1988 for animal experimentation).

**Experiment 1: relationship between oocyte diameter and follicle size**

Immediately after goats were killed, follicles were dissected mechanically from ovaries and transferred to PBS containing sodium pyruvate (0.036 g l¯¹), glucose (1 g l¯¹), penicillin (0.06 g l¯¹) and streptomycin (0.05 g l¯¹) (Gibco BRL, Eragny). Non-atretic follicles were selected on the basis of morphological criteria, such as translucency and the lack of cell debris, and were used for GVBD experiments. Some oocytes were mechanically denuded after culture, and their diameter was measured. Oocytes from each follicle size category were denuded mechanically from cumulus cells and their diameter, excluding the zona pellucida, was measured using a micrometer fitted to an inverted microscope. Fifty oocytes from each category were measured.

**Experiment 2: oocyte growth in vitro**

**Collection of early antral follicles.** Immediately after goats were killed, follicles 0.35–0.45 mm in diameter were isolated from the ovaries at 30–35°C, using a combination of mechanical and enzymatic methods. Small ovarian pieces (1–2 mm) were dissected and placed in 2 ml Hepes-buffered medium 199 (Gibco BRL) (pH 7.35) supplemented with 10% (v/v) fetal calf serum (Dutscher, Brumath), containing 2.5 mg collagenase ml¯¹ (type I, Sigma Chemical, St Quentin-Fallavier), 100 U DNase ml¯¹ (Bovine pancreas III, Sigma Chemical) and 0.15 U pronase XIV ml¯¹ (Sigma Chemical). The ovarian pieces were incubated for 20 min, at 38.5°C, in a rolling culture system, and then washed in M199-Hepes containing 10% fetal calf serum and dissociated with fine forceps under a dissecting microscope. Follicles 0.35–0.45 mm in diameter were recovered and used for the experiment.

**Preparation and culture of granulosa cells.** Granulosa cells were obtained by aspiration of large antral follicles (3-6 mm in diameter). The cells were washed twice in M199. After centrifugation for 4 min at 400g, the supernatant was discarded and the pellet was resuspended in 2 ml M199 buffered with NaHCO₃ containing 100 IU penicillin ml¯¹ (Gibco BRL), 100 μg streptomycin ml¯¹ (Gibco BRL), 250 μg fungizone ml¯¹ (Gibco BRL) and 10% (v/v) fetal calf serum. About 4.5 × 10⁶ cells in 0.5 ml were transferred to tissue culture dishes (Nunc, Poly Labo, Strasbourg). The cells were incubated at 39°C, with 5% CO₂ in humidified air and the medium was replaced every 48 h. The cells were used after they had reached confluence (within 3–5 days).

**Culture of granulosa cell-enclosed oocytes.** Cumulus–oocyte complexes with pieces of parietal granulosa cells (COCGs) were released from the follicles with fine forceps. The diameter of each oocyte, excluding the zona pellucida, was measured and recorded. With oocyte diameters ranging from 78 to 125 μm (mean diameter: 96 μm), the COCGs were divided into two groups according to oocyte diameter, group A: < 95 μm and group B: > 95 μm. Both groups of COCGs were cultured for 8 or 9 days on granulosa cell monolayers, in M199 buffered with NaHCO₃ containing 10% (v/v) fetal calf serum, at 39°C, with 5% CO₂ in humidified air. At intervals of 24 h, half of the medium was replaced. After culture, the COCGs were recovered by mechanical pipetting and the diameter of the oocytes was measured. Oocytes showing evidence of cytoplasmic degeneration and denuded oocytes (4%) were excluded from further analysis.

Some oocytes were mechanically denuded after culture and fixed in ethanol–acetic acid (90%) (3:1, v/v) overnight, at 4°C. They were stained with lacmoid and examined under a microscope to determine their nuclear stage.

**Experiment 3: meiotic competence of oocytes grown in vitro**

The COCGs were isolated and cultured as described in Expt 2. After culture, cumulus–oocyte complexes (COCs) were recovered from COCGs by pipetting, washed in M199 buffered with 20 mmol Hepes 1–¹ (pH 7.35) and matured in vitro, as described by De Smedt et al. (1992). The COCs were cultured in 2 ml M199-Hepes supplemented with 10% (v/v) fetal calf serum, 10 μg FSH ml–¹, 10 μg LH ml–¹ and 1 μg oestradiol ml–¹. Granulosa cells recovered by repeated
pipetting of walls from follicles > 3 mm in diameter were added to the culture medium (1 × 10^6 ml^-1). The COCs were cultured for 27 h in humidified air, at 38.5°C, in a rolling culture system (De Smedt et al., 1992). At the end of the maturation culture, the nuclear status of the oocytes was examined by lacmoid staining, as described above.

Oocytes without evidence of a nucleus or chromosomes were classified as degenerated oocytes.

**Experiment 4: expression of p34^{cd2} in oocytes grown in vitro**

Oocytes grown in vitro were denuded mechanically after culture, and assessed for nuclear status. Oocytes at the GV stage were lysed in sodium dodecyl sulphate (SDS)-sample buffer, and polypeptides were separated using 10% SDS–PAGE as described by Laemmli (1970). Coloured standards. The electrophoretically separated polypeptides were transferred to a nitrocellulose sheet (Schleicher and Schuell, Dassel) according to the method of Towbin (1979), and blocked by incubation in PBS 0.1% Tween-20 (Santa Cruz, CA). The blot was washed three times with PBS 0.1%. Tween-20 and incubated in the same buffer containing the peroxidase-labelled anti-mouse IgG antibody (1:5000) (Biosys, Compiègne). Peroxidase activity was revealed using a blotting chemiluminescence detection kit (Amersham). The intensity of the bands on western blots was measured by a densitometric analysis system (Lecphon Biocom, Les Ulis) and the values were represented graphically.

Equal volumes of oocytes grown in vitro (groups A and B) and of competent oocytes from follicles > 3 mm in diameter used as controls were subjected to immunoblotting. The experiment was repeated three times. In another series of experiments, freshly isolated oocytes from antral follicles < 0.5 mm in diameter and fully grown oocytes from follicles > 3 mm in diameter were processed for immunoblotting as described above.

**Statistical analysis**

Statistical differences in the mean oocyte diameters (Expts 1 and 2) were analysed using Student’s t test. A P value < 0.05 was considered to be significantly different.

**Results**

**Experiment 1: relationship between oocyte diameter and follicle size**

As the antral follicle enlarged up to 3 mm in diameter, the mean oocyte diameter increased progressively from 96 ± 1.5 to 136 ± 0.6 μm (Table 1). The mean oocyte diameter differed significantly among various follicle size classes except between the 2–3 mm and > 3 mm diameter classes, in which oocytes had reached their full size.

**Table 1. Relationship between oocyte diameter and follicle size in goats**

<table>
<thead>
<tr>
<th>Follicle diameter (mm)</th>
<th>Mean ± SEM</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 0.5</td>
<td>96 ± 1.5a</td>
<td>(78–125)</td>
</tr>
<tr>
<td>0.5–0.8</td>
<td>120 ± 0.9b</td>
<td>(109–130)</td>
</tr>
<tr>
<td>1–1.8</td>
<td>125 ± 0.9c</td>
<td>(109–135)</td>
</tr>
<tr>
<td>2–3</td>
<td>136 ± 0.8d</td>
<td>(125–146)</td>
</tr>
<tr>
<td>&gt; 3</td>
<td>136 ± 0.6d</td>
<td>(130–146)</td>
</tr>
</tbody>
</table>

Means with different superscripts differ significantly (P < 0.05).

**Experiment 2: oocyte growth in vitro**

After 8 days of culture on granulosa cells, the mean oocyte diameter increased from 86 ± 0.4 to 95 ± 0.7 μm in group A and from 106 ± 0.2 to 109 ± 0.5 μm in group B (Fig. 1). After a 9 day culture period, the mean diameter of oocytes from groups A and B reached 99 ± 0.5 and 112 ± 0.4 μm, respectively (Fig. 1). The mean diameters of oocytes increased significantly in both groups compared with their values before culture. At the end of the 9 day culture period, 93% of oocytes from group A and 90% of oocytes from group B were at the germinal vesicle stage (GV). Only 1% and 3% of oocytes from groups A and B had undergone GVBD, respectively, while < 10% of oocytes from both groups had degenerated (Table 2).

**Experiment 3: meiotic competence of oocytes grown in vitro**

Meiotic competence of the oocytes was evaluated after in vitro maturation. Within 8 days of culture, only 3% of oocytes from group A and 6% of oocytes from group B acquired GVBD competence (Fig. 2). After the 9 day culture period, 7% of oocytes from group A became competent to undergo GVBD (Fig. 2), half of these oocytes were arrested in prometaphase I and half exhibited abnormal condensed chromatin (Table 3). In contrast, 42% of oocytes from group B acquired the ability to resume meiosis (Fig. 2) (20% arrested in prometaphase I, 3% progressed to metaphase I and 1% to metaphase II, while 18% exhibited a clump of condensed chromatin (Table 3, Fig. 3).

**Experiment 4: p34^{cd2} expression of oocytes grown in vitro**

p34^{cd2} was not detected in freshly isolated oocytes from large antral follicles (Fig. 4a). The presence of p34^{cd2} was analysed by western blotting in both groups of oocytes grown in vitro. Oocytes isolated from large antral follicles

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Table 2. Meiotic stages of goat oocytes cultured for 9 days with granulosa cells

<table>
<thead>
<tr>
<th>Number of oocytes (%)</th>
<th>Cultured</th>
<th>GV</th>
<th>GVBD</th>
<th>Degenerated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>407</td>
<td>378 (93)</td>
<td>5 (1)</td>
<td>24 (6)</td>
</tr>
<tr>
<td>Group B</td>
<td>441</td>
<td>398 (90)</td>
<td>13 (3)</td>
<td>30 (7)</td>
</tr>
</tbody>
</table>

GV, germinal vesicle; GVBD, germinal vesicle breakdown.

Discussion

As reported in mice, pigs and cattle (Sorensen and Wassarman, 1976; Motlik et al., 1984; Fair et al., 1995), goat oocytes become meiotically competent towards the end of their growth phase. Previous experiments showed that goat oocytes sequentially acquired meiotic competence in antral follicles ranging from 0.5 to 2–3 mm in diameter (De Smedt et al., 1994). The present results indicate that the mean oocyte diameter increased from 96 ± 0.3 to 136 ± 0.6 μm as follicle size increased from 0.5 to 2–3 mm. An increase in size from 100 to 120 μm was also observed in the course of meiotic competence acquisition of pig oocytes (Motlik et al., 1984).

Oocytes of diameter < 100 μm from early antral follicles were grown in vitro to render them competent to undergo GVBD. Oocyte growth in mouse oocytes is dependent upon junctional communication between the oocyte and granulosa cells (Eppig et al., 1996). The association between oocytes and granulosa cells was effectively maintained throughout the 9 day culture period of goat COCGs. Beyond this period, most oocytes lost adhesion to granulosa cells (data not shown). This culture system supported oocyte growth, since the mean oocyte diameter increased from 86 ± 0.4 to 99 ± 0.5 μm in group A and from 106 ± 0.2 to 112 ± 0.4 μm in group B within 9 days of culture. Moreover, < 10% of oocytes showed signs of degeneration and most oocytes (90–93%) were still at the GV stage. A collagen gel embedding culture method was used by Harada et al. (1997) for the culture of early antral bovine COCGs. This culture system was efficient in supporting oocyte growth. However, although

(> 3 mm) which express p34cdc2 were used as controls. p34cdc2 was detected after 9 days of culture in oocytes from groups A and B as well as in the control group (Fig. 4b). These data indicate that an accumulation of p34cdc2 occurred during the culture period in both groups of growing oocytes. However, the amount of p34cdc2 increased between group A and group B. The concentration of p34cdc2 was lower in growing oocytes than it was in fully grown oocytes.
the number of oocytes maintaining adhesion to granulosa cells increased in hypoxanthine-supplemented medium, about half of the oocytes were denuded after 7–11 days of culture. Most of these denuded oocytes were degenerated and had resumed meiosis.

Goat oocytes did not acquire meiotic competence within 8 days of culture with granulosa cells. After 9 days of culture, most oocytes from group A remained GVBD-incompetent, but 42% of oocytes from group B had acquired the ability to undergo GVBD. The inability of oocytes from group A to resume meiosis could be related to limited oocyte growth, since the mean oocyte diameter after culture was $99 \pm 0.5 \mu m$.

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### Table 3. Meiotic stages reached after in vitro maturation (27 h) by goat oocytes cultured for 9 days with granulosa cells

<table>
<thead>
<tr>
<th></th>
<th>Number of oocytes resuming meiosis (%)</th>
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<tbody>
<tr>
<td></td>
<td>Examined</td>
</tr>
<tr>
<td>Group A</td>
<td>58</td>
</tr>
<tr>
<td>Group B</td>
<td>110</td>
</tr>
</tbody>
</table>

GV, germinal vesicle; GVBD, germinal vesicle breakdown; cond. ch., condensed chromatin; ProMI, prometaphase I; MI, metaphase I; MII, metaphase II; D, degenerated.

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![Fig. 3. Meiotic stages of goat oocytes after 9 days of culture in vitro. Whole-mounted oocytes were fixed in ethanol–acetic acid and stained with lacmoid. (a) Before in vitro maturation, oocyte at the germinal vesicle (GV) stage. After in vitro maturation (27 h), (b) oocyte that underwent germinal vesicle breakdown, exhibiting a clump of condensed chromatin (arrowhead), (c) oocyte arrested in late prometaphase of the first meiotic division, (d) oocyte in metaphase I, with chromosomes aligned on the metaphase plate of the first meiotic division. Scale bars represent 10 μm.](#)
Most oocytes grown in vitro from early antral follicles were unable to undergo GVBD (De Smedt et al., 1994). The mean diameter of these oocytes was 96 ± 1.5 μm. Oocytes from group B grew to 112 ± 0.4 μm and approximately half of these oocytes acquired GVBD competence. Most oocytes became competent to undergo GVBD in 0.5-0.8 mm follicles (De Smedt et al., 1994) when the oocytes reached 120 ± 0.9 μm in diameter in vitro. Taken together, these results indicate that incomplete growth of oocytes in vitro may be related to restricted oocyte differentiation and, consequently, to limited acquisition of meiotic competence. Half of oocytes grown in vitro that underwent GVBD exhibited abnormal chromatin configuration. These oocytes were probably deficient in molecules controlling nuclear progression. Clumps of condensed chromatin were also found in meiotically competent goat oocytes when matured in the presence of an inhibitor of protein synthesis (Le Gal et al., 1992).

Harada et al. (1997) reported that only a few bovine oocytes acquired meiotic competence after 7 days of culture of COCGs in collagen gel. After an 11 day culture period, 89% of the surviving oocytes underwent GVBD after in vitro maturation and 11% reached MII. In the present study, only 1% of goat oocytes progressed to MII after 9 days of culture. These findings indicate the need for a longer culture period to promote the full meiotic competence acquisition of ruminant oocytes.

As reported by Dedieu et al. (1998), p34<sup>cdc2</sup> was not expressed in GVBD-incompetent goat oocytes derived from early antral follicles, although the presence of cdc2 transcripts was detected in these oocytes. Therefore, a deficiency in the catalytic subunit of MPF that could be regulated at the translational level may account for the incompetence of goat oocytes to resume meiosis. Within the 9 day culture period, p34<sup>cdc2</sup> accumulated in both groups of oocytes growing in vitro. Nevertheless, the amount of p34<sup>cdc2</sup> was lower in growing oocytes than it was in fully grown oocytes isolated from large antral follicles. Although growing oocytes accumulated p34<sup>cdc2</sup> during the culture period, their GVBD competence was still limited, particularly oocytes from group A, in which most oocytes remained arrested at the GV stage. One hypothesis is that p34<sup>cdc2</sup> accumulates in amounts below the threshold required to trigger resumption of meiosis. This hypothesis is supported by the observation that oocytes from group A accumulated less p34<sup>cdc2</sup> than did oocytes from group B. In mice, p34<sup>cdc2</sup> was present in very low amounts in GVBD-incompetent oocytes and accumulated to high amounts when oocytes became competent to undergo GVBD (Chesnel and Eppig, 1995; de Vantéry et al., 1996). However, GVBD-incompetent mouse oocytes accumulated p34<sup>cdc2</sup> when cultured in vitro under conditions that did not support acquisition of GVBD competence (Chesnel and Eppig, 1995). The same situation was observed in goat oocytes and another hypothesis is that the accumulation of p34<sup>cdc2</sup>, although necessary, is only one of the factors controlling the acquisition of GVBD competence. The accumulation of additional factors involved in the MPF regulatory machinery is probably also required for the oocytes to become GVBD competent. Several cell-cycle related molecules involved in meiotic progression remain to be investigated in ruminant oocytes, and further studies are required to gain more information on the molecular basis of the acquisition of meiotic competence.

In conclusion, the present study indicated that the culture of granulosa cell-enclosed oocytes isolated from early antral follicles for 9 days supported oocyte growth and the acquisition of GVBD competence to a certain extent. Extending the culture period may be necessary to promote full meiotic competence and to confer to a large number of oocytes the ability to resume meiosis and complete meiotic maturation. Developing culture systems that support full oocyte development and differentiation would require defining the conditions for producing oocytes that express RNA and protein patterns close to those of oocytes grown in vitro.

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