

Follicular size affects the meiotic competence of in vitro matured prepubertal and adult oocytes in sheep

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Abstract — Non-atretic follicles dissected from prepubertal and adult ovaries were allocated in three groups: a) < 1 mm; b) 1–2 mm; c) > 2 mm. After 24 h of maturation, a lower percentage of adult oocytes from group a ($P < 0.01$) reached metaphase II than those from groups b and c (70.4 versus 89.5 and 95.5). Prepubertal oocytes showed similar results ($P < 0.01$; 27.2 versus 79.8 and 81.8). There was a significant difference ($P < 0.01$) in meiotic progression between prepubertal and adult oocytes of < 1 mm follicles. The diameter of prepubertal oocytes derived from group a was significantly lower ($P < 0.01$) compared to groups b and c (138.1 versus 142.1 and 145.6); adult oocytes showed similar results ($P < 0.01$; 142.2 versus 157.2 and 158.1). Oocytes with the same diameter derived from different follicles of prepubertal and adult ovaries showed similar meiotic progression rates. © Inra/Elsevier, Paris

ovary / oocyte / follicle size / meiotic competence / adult and prepubertal sheep

Résumé — Lien entre la taille et la compétence méiotique d'ovocytes d'agnelles et de brebis matures in vitro. Des follicules non-atrétiques sélectionnés à partir d'ovaires d'ovins prépubères et adultes ont été répartis en trois groupes selon leur diamètre : a) < 1 mm ; b) 1–2 mm ; c) > 2 mm. Après 24 h de maturation les ovocytes dérivant des ovaires d'animaux adultes appartenant au groupe a ont atteint la MII dans des pourcentages nettement inférieurs ($p < 0,01$) à ceux observés pour les groupes b et c (70,4 versus 89,5 et 95,5). Des résultats identiques ont été obtenus avec les ovocytes provenant d'animaux prépubères ($p < 0,01$; 27,2 versus 79,8 et 81,8). Une grande différence ($p < 0,01$) dans le potentiel à reprendre la méiose existe entre les ovocytes de prépubères et des adultes provenant de follicules < 1 mm. Le diamètre des ovocytes d'agneaux provenant de follicules du groupe a était nettement inférieur ($p < 0,01$) par rapport à ceux provenant de follicules des groupes b ou c (138,1 versus 142,1 et 145,6). Des résultats identiques ont été observés pour les ovocytes provenant d'animaux adultes ($p < 0,01$, 142,2 versus 157,2 et 158,1). Des ovocytes de même diamètre provenant d'ovaires de prépubères et d'adultes ne diffèrent pas dans leur aptitude à reprendre la méiose. © Inra/Elsevier, Paris

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1. INTRODUCTION

It is known that mammalian oocytes released from the inhibitory influences of their follicles and cultured *in vitro* under efficient conditions are able to spontaneously resume meiosis and complete maturation [10]. Several data indicate, however, that many of these oocytes fail to be fertilised, develop or produce a high percentage of viable embryos [12, 18, 29]. During oocyte growth, physiological events occur in both the nucleus and the cytoplasm allowing the oocyte to achieve final developmental competence. Nuclear progression from the germinal vesicle (GV) to the metaphase II (MII) stage can occur in oocytes that may not have undergone complete cytoplasmic maturation [2]. Many intracellular changes are required in the cytoplasm, such as organelle distribution [28, 29] and protein synthesis at different stages of meiosis progression [15, 22]. Moreover, the meiotic competence in bovine species is closely related to the follicular dimension; oocytes derived from small follicles showed a lower capacity to resume meiosis and to develop up to early embryonic stages [4]. Previous works [8, 19, 23] have clearly demonstrated that the acquisition of meiotic competence after *in vitro* maturation is achieved progressively as the follicular size increases in several domestic species. Oocytes derived from follicles less than 2 mm in diameter did not resume or complete meiosis *in vitro*.

In sheep, Moor and Trounson [21] also indicated that meiotic and developmental competence is higher in oocytes recovered from large follicles (atretic and non-atretic) than in those derived from small follicles. They also showed that oocytes cultured in intact follicles (< 2 mm) are incapable of resuming meiosis. A positive correlation between follicular size and meiotic and developmental competence has also been reported by Cogniè et al. [5]. They showed that a different genotype can affect the ability of oocytes recovered from follicles of different sizes to resume meiosis *in vitro*.

A lower developmental capacity has also been observed after *in vitro* maturation of oocytes obtained from prepubertal animals [9, 26, 27]. This can be related to defective conditions of oocyte maturation and can also be due to the reduced size of oocytes and follicular population [17] and a small size of volume fractions of mitochondria and cortical granules [25].

The aim of this work was to examine the relationship between the follicular size and meiotic competence of oocytes derived from prepubertal and adult sheep in different categories of follicles after *in vitro* maturation.

2. MATERIALS AND METHODS

2.1. Recovery of the oocytes

Ovaries from prepubertal (30–40 days, body mass 6–12 kg) and adult Sarda sheep were collected from a local abattoir and transported in Dulbecco's phosphate-buffered saline (PBS; Sigma, Saint-Louis, MO) containing 50 U penicillin mL⁻¹ and 50 µg streptomycin mL⁻¹ (SIGMA) at 20–25 °C, arriving at the laboratory within 1 h. Ovaries were washed three times in fresh PBS with penicillin and streptomycin.

Non-atretic follicles were obtained from the ovaries using microblades and fine forceps under a dissecting microscope and single selected follicles were obtained from both prepubertal and adult ovaries. After dissection, the follicles were measured with a micrometer and placed according to diameter in the three following groups: a) < 1 mm; b) 1.0–2.0 mm; c) > 2 mm. The walls of the follicles of the different groups were opened and the oocytes were released in the TCM 199 medium (with Earle's salts and bicarbonate; Sigma) supplemented with 25 mmol Hepes I⁻¹ (Sigma), penicillin and streptomycin, and 0.1 % (w/v) polyvinyl alcohol (PVA; Sigma). The cumulus–oocyte complexes (COCs), selected according to morphological criteria (intact COCs with several dense cumulus cell layers and homogeneous cytoplasm), were released in the medium and washed three times in the same medium before *in vitro* maturation.

After denudation of the cumulus cells by mechanical pipetting, several oocytes obtained from each follicle-sized group of prepubertal and adult sheep, including the zona pellucida, were

measured using an inverted microscope equipped with a calibrated eyepiece. Their outside diameters were recorded.

2.2. In vitro maturation

The COCs obtained from juveniles were allocated to the following maturation system: prepubertal and adult oocytes were cultured in parallel for 24 h in TCM 199 medium supplemented with 10 % (v/v) heat-treated foetal calf serum (FCS; Sigma) in the presence of 10 µg FSH mL⁻¹ and 10 µg LH mL⁻¹ (Pergonal, Serono, Rome). Only 25–30 of the oocytes were put in a single Petri dish (35 mm) containing 2 mL of the culture medium which was maintained on a rotary shaker at low speed during the entire incubation period to avoid the attachment of granulosa cells to the bottom of the Petri dish. The culture conditions were the following: a humidified atmosphere with 5 % CO₂ in the air and a temperature of 39 °C. At the end of the maturation period, the oocytes from each group were fixed for 24 h in acetic acid ethanol solution (1:3, v/v), stained with 1 % (w/v) Lacmoid (Sigma) and examined under a phase-contrast microscope at a magnification of × 200–400. The stages of nuclear maturation were assessed after evaluation, as the germinal vesicle (GV), metaphase I (M I), metaphase II (M II) and degenerated.

Some oocytes from prepubertal ovary < 1 mm follicles were cultured for 48 h in the same culture conditions as those described above, examined after 24 h of fixation and stained with 1 % Lacmoid.

2.3. Statistical analysis

Differences in meiotic progression according to the follicular size of prepubertal and adult oocytes were subjected to chi-squared analysis or the Fisher exact test. Mean oocyte diameters in the follicle size categories were compared statistically by ANOVA. Differences were considered significant at $P < 0.05$.

3. RESULTS

After 24 h of incubation the percentage of metaphase II oocytes derived from ovaries of adult and prepubertal animals increased progressively according to follicle sizes (from < 1 to > 2 mm (*table I*). There was a significant difference ($P < 0.01$) in the percentage of M II between oocytes derived from < 1 mm follicles compared to oocytes of > 1 and > 2 mm of both prepubertal (27.2 versus 79.8 and 81.8 %) and adult (70.6 versus 89.7 and 95.5 %) sheep. Oocytes collected from < 1 mm follicles of prepubertal animals also showed a significantly ($P < 0.01$) marked decrease in the ability to resume complete meiosis compared to adult oocytes. This difference can be related to a significantly lower ($P < 0.01$) oocyte diameter in the three categories of follicles (< 1, 1–2 and > 2 mm) of prepubertal (138.1, 142.1 and 145.6 µm) compared to adult sheep (142.2, 157.2 and 158.1 µm) (*table II*).

Table I. Meiotic competence of prepubertal and adult oocytes derived from follicles of different size.

	Follicle size (mm)	Oocyte number	Stage of meiotic progression in adult oocytes			
			GV (%)	M I (%)	M II (%)	Deg. (%)
Adults	< 1.0	85	10 (11.7) ^a	10 (11.7) ^a	60 (70.6) ^a	5 (5.4)
	1.0–2.0	88	– ^b	9 (10.2) ^b	79 (89.7) ^b	–
	> 2.0	45	– ^b	2 (4.5) ^b	43 (95.5) ^b	–
Prepubertal	< 1.0	92	25 (27.2) ^c	37 (40.2) ^c	25 (27.2) ^c	5 (5.4)
	1.0–2.0	94	6 (6.4) ^d	10 (10.6) ^a	75 (79.8) ^b	3 (3.2)
	> 2.0	44	3 (6.8) ^d	4 (9.1) ^a	36 (81.8) ^b	1 (2.3)

Between columns, test χ^2 : a versus b = $P < 0.01$; a,b versus c = $P < 0.001$; c versus d = $P < 0.01$.

Table II. Oocyte diameter of prepubertal and adult sheep derived from follicles of different sizes.

Oocyte	Follicle size in both prepubertal and adult sheep		
	< 1 mm	1–2 mm	> 2 mm
Adult	142.2 ± 3.5 µm ^a	157.2 ± 2.9 µm ^b	158.1 ± 3.1 µm ^b
Prepubertal	138.1 ± 2.6 µm ^c	142.1 ± 3.1 µm ^a	145.6 ± 2.4 µm ^d

Test ANOVA: different superscripts are statistically different ($P < 0.01$).

Oocytes derived from < 1 mm follicle size cultured in vitro for 48 h showed a similar percentage of M II as those cultured for 24 h (29.7 versus 27.2).

4. DISCUSSION

This work shows that the ability to progress in the meiotic cycle is achieved progressively as follicle and oocyte diameter increases in both adult and prepubertal sheep. However, in contrast to the observations of De Smedt et al. [8] in goats, sheep oocytes derived from very small follicles (< 1 mm) of adult ovaries can progress in meiosis in a high percentage and 70 % of total oocytes reach metaphase II.

Several studies in other domestic species [6, 11, 23], indicate that significantly more oocytes derived from > 2 mm follicles matured in vitro up to M II, while oocytes collected from follicles of less than 2 mm are incapable of resuming or completing meiosis. In prepubertal goats it has been reported [20] that complete meiotic competence was achieved in oocytes derived from 3 mm in diameter follicles and similar results have been obtained in adult goats by De Smedt et al. [8]. They observed that the oocyte was able to resume meiosis in < 1 mm antral follicles but only oocytes derived from follicles larger than 3 mm were fully competent and able to reach metaphase II. These authors also showed that oocytes from 2 mm follicles were still actively engaged in RNA synthesis, whereas RNA synthesis was

reduced in oocytes from 3 mm follicles. Motlik et al. [24] reported that porcine oocytes derived from < 0.7 mm follicles remained at the germinal vesicle stage maintaining activity in RNA synthesis. The capacity to resume meiosis also seems to be related to the location of the follicles in the ovaries. Arlotto et al. [1] reported that oocytes of all diameters collected from the cortical region of the ovary are significantly more competent than oocytes located in a sub-cortical area. More recently Hyttel et al. [14] showed a direct correlation only between bovine oocyte dimension and meiotic developmental competence after the maturation of oocytes derived from follicles of different sizes.

In sheep, after the culture of intact follicles of different sizes, Moor and Trounson [21] showed that oocytes derived from < 2 mm follicles were meiotically incompetent while on the contrary oocytes from bigger follicles were meiotically and developmentally fully competent. This is apparently in contrast with the results reported in this paper but our culture conditions were different and oocytes were cultured outside of the follicles and not in intact follicles as Moor and Trounson reported. These conditions may have allowed the oocyte to progress to meiosis because it cannot be influenced by the inhibitory control of meiosis exerted by the follicular environment. Cogniè et al. [5] also found that the ability of ovine oocytes to resume meiosis and to gain the ability to develop up to the blastocyst stage is acquired sequentially as the follicle

enlarges. They also showed that this ability is related to the genotype of the ewe. In fact, oocytes from < 1 mm follicles of *Fec^B* gene carriers were able to resume meiosis in higher percentages than those from non-carriers. They also found that the pattern of oocyte growth in preantral follicles was related to the genotype and that *Fec^B* carriers had larger oocytes than those from non-carriers. Our data showed that in Sarda ewes the ability to resume meiosis is high from oocytes derived from < 1 mm follicles and may be related to the genotype of this breed. In fact the oocytes derived from these follicles, which are even smaller than those from larger follicles, are capable of being meiotically competent.

In contrast, the experiment of maturation of prepubertal oocytes indicated that oocytes derived from < 1 mm follicles were incapable of resuming and completing meiosis compared to adult oocytes (27 versus 70 of M II) and a higher percentage of the oocytes remained arrested at the GV and M I stage. This difference in meiotic progression may be due to the smaller oocyte diameter in prepubertal oocytes (138 versus 142 μm) probably indicating an incomplete growth phase. The acquisition of meiotic competence may be closely related to oocyte diameter because at 142 μm in diameter both prepubertal and adult sheep oocytes show similar nuclear progression (79.8 versus 70.6). In fact, bovine oocytes derived from small follicles showed a low diameter of < 100 μm and are unable to resume meiosis or are arrested at M I; moreover these oocytes show an intense transcriptional activity [11].

In prepubertal oocytes the lower capability to resume and complete meiosis can be due to either inefficient coupling between the somatic compartment and the oocyte that can decrease the signals involved in meiotic resumption or the lack of key molecules regulating the meiosis cycle. In fact, it has been observed that the gap junctions increase with the follicular dimensions [13]. Prepubertal oocytes have also shown a

less functional coupling between the corona cell and oocytes [16]. The increase in gap junctions between somatic cells and the oocyte is also related to the follicular dimension during the folliculogenesis. Furthermore, it has been observed that growing oocytes are still engaged in high RNA synthesis that may be crucial for the production of molecules directly involved in cell cycle progression. In fact, it has been observed [3] that prepubertal ovine oocytes showed a lower H1 activity and a lower MPF biological activity after fusion of M II prepubertal oocytes with GV stage oocytes. Similarly it has been reported that calf oocytes had lower H1 and MAPK activities compared to adult oocytes [7]. This lower competence in prepubertal oocytes has also been observed after fertilisation and culture *in vivo* and *in vitro* up to the blastocyst stage [17].

In conclusion, these experiments showed that, contrary to observations in prepubertal sheep, a high percentage of adult oocytes derived from very small follicles are able to reach full meiotic progression. Meiotic competence may, however, be closely related to oocyte diameter in sheep.

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