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Microtubule and microfilament organization in immature, *in vitro* matured and *in vitro* fertilized prepubertal goat oocytes

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Summary

The aim of our study was to analyse the cytoskeletal organization of prepubertal goat oocytes. Microtubule and microfilament organization during *in vitro* maturation of prepubertal and adult goat oocytes and presumptive zygotes of *in vitro* matured–*in vitro* fertilized (IVM-IVF) prepubertal goat oocytes were analysed. Oocytes were matured in M-199 with hormones and serum and inseminated with frozen-thawed spermatozoa. Oocytes and presumptive zygotes were treated with anti-α-tubulin antibody and fluorescein isothiocyanate (FITC)-labelled goat anti-mouse antibody to stain the microtubules. Microfilaments were localized by means of phalloidin 5 μg/ml conjugated with fluorescein isothiocyanate (FITC-phalloidin). DNA was stained with propidium iodide. Stained oocytes were observed under a confocal laser scanning microscope. At the germinal vesicle nuclear stage, microfilaments were distributed at the cortex of the oocytes. After *in vitro* maturation, 91.7% of metaphase II (MII) oocytes from adult goats displayed microfilaments in the cortex and within the polar body and were characterized by the presence of a microfilament thickening at the cortical region over the meiotic spindle. In prepubertal goat MII oocytes only 5.7% of oocytes displayed microfilaments at the cortex and within the polar body. After insemination, most of the zygotes displayed microfilaments distributed at the cortex. An undefined microtubular network was observed in adult and prepubertal goat oocytes at the germinal vesicle stage. After *in vitro* maturation, 100% of MII oocytes from adult goats displayed microtubules on the meiotic spindle and within the polar body. This pattern of distribution was observed in 71.6% of prepubertal goat oocytes. Undefined microtubule networks were present in most of the zygotes analysed. In conclusion, cytoskeletal differences were found between prepubertal and adult goat MII oocytes. Furthermore, most of the zygotes from IVM-IVF prepubertal goat oocytes displayed cytoskeletal anomalies.

Keywords: Goat, IVM, Microfilaments, Microtubules, Oocytes

Introduction

The cytoskeleton is a complex network of two major elements: microtubules and microfilaments. Microtubules and microfilaments play a significant role during oocyte maturation and fertilization. Microfilaments are a network of actin filaments that make up the cell cortex, giving strength to the surface of the cell and determining its shape and polarity. Microfilaments are involved in chromosome and spindle maintenance, in peripheral spindle localization, in polar body extrusion (Webb *et al.*, 1986; Kim *et al.*, 1996*a*–*c*) and in pronuclear apposition during fertilization in pig (Kim *et al.*, 1997*a*, *b*) and mouse (Maro *et al.*, 1984) oocytes. Microfilaments are also reported to be involved in the distribution of mitochondria (Barnett *et al.*, 1997; Boldogh *et al.*, 1998), in Golgi complex distribution and morphology (Valderrama *et al.*, 1998), in ionic regulation (Cantiello, 1997) and in mRNA positioning (Basell *et al.*, 1994). On the other hand, microtubules support the structures and play a fundamental role in

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the formation of meiotic structures and migration of cytoplasmic organelles. Microtubules are responsible for chromosome reorganization and migration and for mitochondrial positioning in human (Pickering *et al.*, 1988; Kim *et al.*, 1998), mouse (Van Berklom, 1986; Messinger & Albertini, 1991), cattle (Aman & Parks, 1994), pig (Sun *et al.*, 2001) and horse (Tremoleda *et al.*, 2001).

In most mammalian oocytes, the distribution of microtubules and microfilaments changes dramatically during meiosis. Concerning their location, microfilaments are concentrated in the cell cortex during meiosis, although a secondary aggregation has been described overlying the meiotic spindle in order to maintain the spindle and the chromosomes in a peripheral position in human (Kim *et al.*, 1998) and pig (Sun *et al.*, 2001). Microtubule distribution differs among species. In mouse, microtubules are distributed throughout the cytoplasm during interphase; in metaphase they are mostly found in association with the spindle-associated centrosomes (Messinger & Albertini, 1991). In other mammalian species cytoplasmic microtubules are only detected associated with the metaphase plate in sheep (Le Guen & Crozet, 1989), rabbit (Yllera-Fernandez *et al.*, 1992), pig (Kim *et al.*, 1996*a*) and human (Kim *et al.*, 1998).

It has been shown in *in vivo* and *in vitro* studies that adverse oocyte culture conditions could affect actin microfilament polymerization (Matsumoto *et al.*, 1998) and microfilament network structures (Wang *et al.*, 1999), consequently affecting embryonic development. Moreover, it has been reported that *in vitro* aged oocytes (Kim *et al.*, 1996*b*) showed an abnormal organelle distribution in the zygote that can impair further embryonic development.

Oocytes from prepubertal females show lower embryo development than oocytes from adult females (reviewed by Armstrong, 2001). In our laboratory, the percentage of blastocysts obtained from prepubertal goats is low (10%) (Izquierdo *et al.*, 1999; Rodriguez-Gonzalez *et al.*, 2003) compared with that obtained from oocytes of adult goats. Thus, Cognié (1999) obtained 36% blastocysts, Crozet *et al.* (1995) 26% and Keskintepe *et al.* (1996) 32% using adult goat oocytes. Armstrong (2001), comparing embryo development of prepubertal and adult female oocytes, concluded that calf oocytes exhibited abnormal chromatin and microtubule configuration characterized by delayed formation of the sperm aster and asynchronous pronuclear formation. Unlike the findings with calf oocytes, no differences were seen in intracellular distribution of organelles between oocytes from prepubertal lambs and adult ewes. The last conclusion of this author is that oocytes from prepubertal females are less robust and may be less tolerant to suboptimal culture conditions than oocytes from adult females. It has been proposed that inadequate culture conditions during *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) could also impair the function of cytoplasmic organelles, including microtubule and microfilament assembly, which could result in abnormal pronuclear formation and in a lower rate of early embryonic development (Hyttel *et al.*, 1999). Moreover, in contrast to *in vivo* matured bovine oocytes, *in vitro* matured–*in vitro* fertilized bovine oocytes showed abnormal spindle localization and abnormal pronuclear apposition (Long *et al.*, 1993, 1994). As far as we know, no studies have reported on a comparison of microtubule and microfilament distribution during IVM of prepubertal and adult goat oocytes.

The aim of the study was to describe microtubule and microfilament distribution of prepubertal goat oocytes at oocyte retrieval (0 h of IVM), after 27 h of IVM and at 20 h post-insemination.

Materials and methods

Reagents

Tissue culture plasticware was purchased from Falcon Labware (Lincoln Park, USA) and Nunc (Roskilde, Denmark) and all reagents were from Sigma Chemical (St Louis, MO) unless otherwise stated. Steer serum (Donor Bovine Serum) was from CanSera (Ontario, Canada), ovine FSH (Ovagen) from Immuno Chemical Products (Auckland, New Zealand), and LH was kindly supplied by Dr J.F. Beckers (IRSIA Research Unit, University of Liege, Belgium). Dulbecco's phosphate-buffered saline (PBS) and PBS modified by the addition of 1000 mg/l glucose and 36 mg/l pyruvate (mPBS) were purchased from Gibco (Paisley, UK).

Collection of adult and prepubertal goat oocytes and *in vitro* maturation

Ovaries from prepubertal goats, approximately 2 months old, and adult goats (more than 3 years old) were obtained from a local slaughterhouse and transported to the laboratory within 2 h of slaughter, at 37 °C in Dulbecco's PBS containing 50 μg gentamicin/ml. The ovaries were washed three times in PBS, and the follicular contents recovered by aspiration of follicles, 2–6 mm in diameter, through a 19-gauge needle under constant vacuum. The cumulus–oocyte complexes were released into HEPES-buffered TCM199 medium, supplemented with heparin 10 μg/ml (170 USP/mg) and 50 μg gentamicin/ml. Only oocytes with one or more completed layers of unexpanded cumulus cells and evenly granulated cytoplasm were used. They were washed three times

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in HEPES-buffered TCM199 medium and once in the maturation medium. Groups of 25–40 cumulus-enclosed oocytes were transferred to 4-well tissue plates containing 500 µl of maturation medium for 27 h at 38.5 °C in an atmosphere of 5% CO_2 in air with maximum humidity. The maturation medium was TCM199 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 10 µg LH/ml, 10 µg FSH/ml and 1 µg 17β-estradiol/ml. Samples of oocytes were stained just after IVM.

In vitro fertilization

At the end of the IVM period, goat oocytes were inseminated to investigate their fertilization competence. Sperm capacitation and oocyte insemination were performed as described by Huneau & Crozet (1989) and modified in our laboratory. Briefly, frozen-thawed sperm cells from 4 males of proven fertility were pooled and prepared by centrifugation through a 90% and 45% discontinuous Percoll gradient at 700 *g* for 20 min. After centrifugation, the pellet was resuspended in a modification of the defined medium with Hepes (DMH), as described by Brackett & Oliphant (1975). The sperm pellet was diluted and maintained in DMH supplemented with 20% (v/v) steer serum for at least 1 h at 38.5 °C. Sperm cells were then diluted in the fertilization medium, composed of DMH, 20% steer serum and 7.75 mM calcium lactate, to inseminate with a final sperm concentration of 2×10^6 sperm cells/ml. Sperm and oocyte co-incubation was performed in tubes at 38.5 °C for 20 h (the time at which two pronuclei could be easily observed by Hoechst staining in prepubertal goat oocytes; Velilla *et al.*, 2002).

Immunostaining

Microtubules and microfilaments were stained as described by Kim *et al.* (1996*a*) with some modifications. Immunostaining of microtubules and microfilaments were done separately because in our material triple staining gave us a lower-quality signal and more background. Just after oocyte collection, after 27 h of IVM and at 20 h after IVF, oocytes were fixed in 4% paraformaldehyde for 30 min and permeabilized in 25% glycerol, 50 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM EGTA, 1 mM 2-mercaptoethanol, 50 mM imidazole, pH 6.7, with 1% Triton X-100. After that, oocytes were rinsed three times, 5 min each time, with blocking medium (PBS with bovine serum albumin (BSA) 0.1% (w/v) and glycine 7.5 mg/ml).

Microtubules were localized by use of monoclonal anti-α-tubulin antibody (Ab, Sigma) diluted 1:50 in PBS for 45 min. After three washes in PBS, 10 min each time, oocytes were incubated in fluorescein isothiocyanate (FITC)-labelled goat anti-mouse antibody (FITC conjugate antimouse IgG, Sigma) diluted 1:150 in PBS for 45 min at 38 °C under dark conditions. Microfilaments were localized by means of phalloidin 5 µg/ml conjugated with fluorescein isothiocyanate (FITC-phalloidin, Sigma).

Stained oocytes were washed three times in PBS, 10 min each time. DNA was observed by exposure to 20 µg/ml propidium iodide (Sigma) and, after several washes in PBS, the oocytes were mounted between a polylysine-coated coverslip and a glass slide supported by four columns of paraffin. The slide was sealed with nail polish. The control group consists of prepubertal goat oocytes fixed and stained with propidium iodide. Stained oocytes were observed under a confocal laser scanning microscope (Leica TCS 4D equipped with a krypton–argon ion laser and Pl fluotar 16×/0.50 IMM, Pl fluotar 40×/1.00–0.50 OIL, Pl APO 63×/1.40 OIL, Pl fluotar 100×/1.30–0.60 OIL lenses).

Two control groups (10 oocytes per group) were used in order to assess that no staining meant absence of microtubules and microfilaments. In the first the secondary antibody (FITC-labelled goat anti-mouse antibody) for microtubules was omitted and in the second phalloidin staining for microfilaments was omitted.

Evaluation and classification of microfilaments:

C: Microfilaments located in the cortex.
C + P: Microfilaments located in the cortex and also polymerized within the cytoplasm.
CC + PB: Microfilaments located in the cortex with an actin-rich domain overlying the metaphase spindle and within the polar body.
C + PB: Microfilaments located in the cortex and within the polar body.
C + PN: Microfilaments located in the cortex and around the pronuclei.
C + Z: Microfilaments located in the cortex and around the sperm head.

Evaluation and classification of microtubules:

UN: Undefined microtubule organization.
PN: Tubulin localized around the pronuclei.
S: Microtubules localized in the spindle.
S + PB: Microtubules localized in the spindle and within the polar body.
PB: Microtubules localized within the polar body.
Z: microtubules around the condensed sperm head.

Statistical analysis

A statistical evaluation of the differences in microfilament and microtubule distribution between prepubertal and adult goat oocytes at 0 and after 27 h of IVM was carried out. The statistical analysis used was Fisher's exact test. Data were considered significantly different at $p < 0.05$.

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Table 1 Microfilament organization according to nuclear stage of adult goat oocytes at 0 and 27 h after *in vitro* maturation

Nuclear stage			Microfilament organization									
			C *n* (%)		C + P *n* (%)		CC + PB *n* (%)		C + PB *n* (%)		Unstained *n* (%)	
0 h of IVM ($n = 27$)												
GV	19	(70.4)	15	(78.9)	–	–	–	–	–	–	4	(21.1)
MI	7	(25.9)	7	(100.0)	–	–	–	–	–	–	–	–
MII	1	(3.7)	1	(100.0)	–	–	–	–	–	–	–	–
27 h of IVM ($n = 32$)												
GV	1	(3.1)	1	(100.0)	–	–	–	–	–	–	–	–
MI	7	(21.9)	3	(42.9)	–	–	4	(57.1)	–	–	–	–
MII	24	(75.0)	–	–	2	(8.3)	22	(91.7)	–	–	–	–

GV, germinal vesicle; MI, metaphase I; MII, metaphase II; C, cortex; C + P, cortex and polymerized within the cytoplasm; CC + PB, cortex with an actin-rich domain overlaying the metaphase spindle and within the polar body; C + PB, microfilaments located in the cortex and within the polar body.

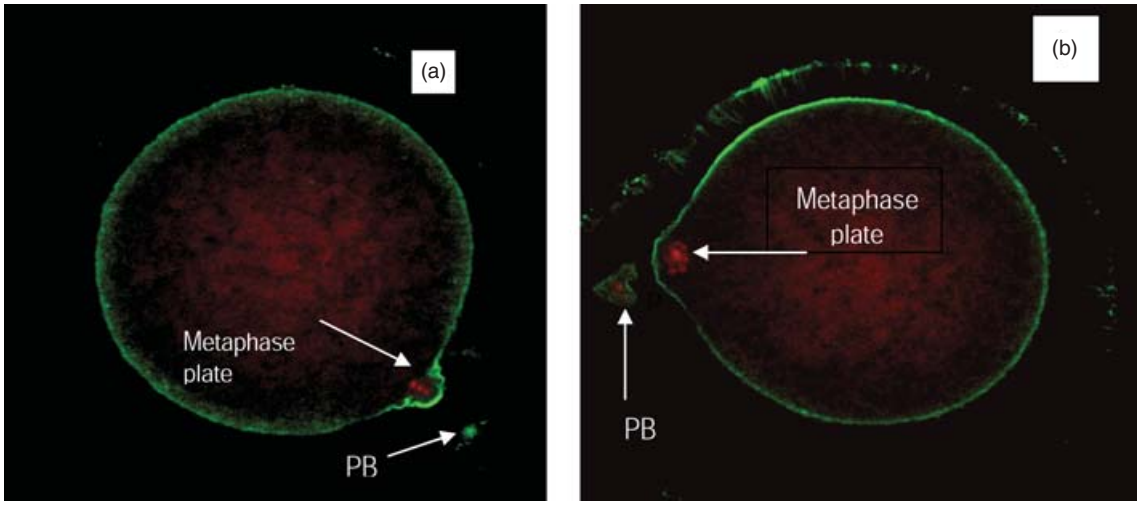

Figure 1 Microfilament distribution in goat oocytes. (a) MII stage adult goat oocytes. Microfilament thickening (green) is seen around the metaphase plate (red). (b) MII stage prepubertal goat oocytes. Microfilaments are organized at the cortex and inside the polar body.

Results

Microfilament distribution

Microfilament distribution at 0 and 27 h of IVM in adult and prepubertal goat oocytes

Table 1 presents the results of microfilament distribution in adult goat oocytes. Most (78.9%) of the oocytes at the germinal vesicle (GV) stage displayed microfilaments at the oocyte cortex. After 27 h of IVM, 91.7% of oocytes at the MII stage presented microfilaments at the cortex and within the polar body and were characterized by the presence of a microfilament thickening at the cortical region over the meiotic spindle (Fig. 1a).

Table 2 shows the results of microfilament organization of prepubertal goat oocytes. Just after oocyte collection, 54% of the oocytes have resumed meiosis before culture for IVM. No difference in microfilament distribution was observed between oocytes at GV stage (0 h) and at metaphase II (MII) (27 h of IVM). Only 5.7% of MII oocytes displayed microfilaments at the cortex and within the polar body (Fig. 1b), but there was no microfilament thickening over the meiotic spindle as was observed in the oocytes of adult goats.

Statistically significant differences between adult and prepubertal goat oocytes were found in the microfilament distribution in the cortex of GV stage oocytes (78.9% and 98.3%, respectively; $p < 0.01$) and

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Table 2 Microfilament organization according to nuclear stage of prepubertal goat oocytes at 0 and 27 h after *in vitro* maturation

Nuclear stage	N (%)	Microfilament organization									
		C *n* (%)		C + P *n* (%)		CC + PB *n* (%)		C + PB *n* (%)		Unstained *n* (%)	
0 h of IVM ($n = 129$)											
GV	59 (45.7)	58	(98.3)	1	(1.7)	–	–	–	–	–	–
MI	68 (52.7)	47	(69.1)	20	(29.41)	–	–	–	–	1	(1.5)
MII	2 (1.5)	1	(50.0)	1	(50.0)	–	–	–	–	–	–
27 h of IVM ($n = 124$)											
GV	7 (5.6)	7	(100.0)	–	–	–	–	–	–	–	–
MI	10 (8.1)	10	(100.0)	–	–	–	–	–	–	–	–
A/T	1 (0.8)	1	(100.0)	–	–	–	–	–	–	–	–
MII	1106 (85.5)	89	(83.9)	11	(10.4)	–	–	6	(5.7)	–	–
								6			

GV, germinal vesicle; MI, metaphase I; A/T, anaphase/telophase; MII, metaphase II; C, cortex; C + P, cortex and polymerized within the cytoplasm; CC + PB, cortex with an actin-rich domain overlaying the metaphase spindle and within the polar body; C + PB, microfilaments located in the cortex and within the polar body.

Table 3 Microfilament organization according with nuclear stage of prepubertal goat IVM-IVF-oocytes at 20 h post insemination ($n = 93$)

Nuclear stage	*n* (%)	Microfilament organization							
		C *n* (%)		C + P		C + PN		C + Z	Unstained *n* (%)
MII	13 (14.0)	6	(46.2)	7	(53.8)	–	–	–	– –
MII+ Z	5 (5.4)	3	(0.6)	–	–	–	–	–	2 (0.3)
1PN	1 (1.1)	1	(100)	–	–	–	–	–	– –
1PN+Z	8 (8.6)	7	(87.5)	–	–	–	–	1 (12.5)	– –
2PNs	24 (25.8)	21	(87.5)	–	–	3	(12.5)	–	– –
2Pna	18 (19.3)	12	(66.7)	–	–	5	(27.8)	–	– 1 (5.5)
PS	24 (25.8)	17	(70.8)	2	(8.3)	–	–	4 (16.7)	1 (4.2)

MII, metaphase II; MII + Z, metaphase II and a sperm head; 1PN, one pronucleus; 1PN + Z, one pronucleus and one sperm head; 2PNs, two synchronous pronuclei; 2PNa, two asynchronous pronuclei; PS, polyspermic; C, cortex; C + P, cortex and polymerized within the cytoplasm; C + PN, cortex and polymerized around pronuclei; C + Z, cortex and polymerized around the sperm head.

in MII microfilament distribution CC + PB (91.7% and 0, respectively; $p < 0.0001$).

Microfilament distribution in zygotes of IVM-IVF prepubertal goat oocytes

Table 3 shows the microfilament distribution of 93 prepubertal goat oocytes at 20 h post-IVF. The percentage of fertilized oocytes was 85% (79/93). Twenty-nine per cent (24/93) of oocytes were correctly fertilized (oocytes with two synchronous pronuclei).

Of the 79 fertilized oocytes, 60 showed microfilaments at the cortex, 8 showed, apart from cortical microfilaments, some polymerized microfilaments around pronuclei and 5 displayed cortical microfilaments and some polymerized microfilaments localized around the sperm heads.

Actin projections from cumulus cells into the oocyte were observed in both prepubertal and adult goat oocytes in a similar pattern.

Microtubule distribution

Microtubule distribution at 0 and 27 h of IVM in adult and prepubertal goat oocytes

Table 4 presents the results of the analysis of microtubule organization in adult goat oocytes. Just after collection, 100% of oocytes at the GV stage showed an undefined microtubule network. After IVM, 100% of the oocytes at the MII stage showed microtubules, which were distributed at the meiotic spindle and within the polar body (Fig. 2a). The meiotic spindle was

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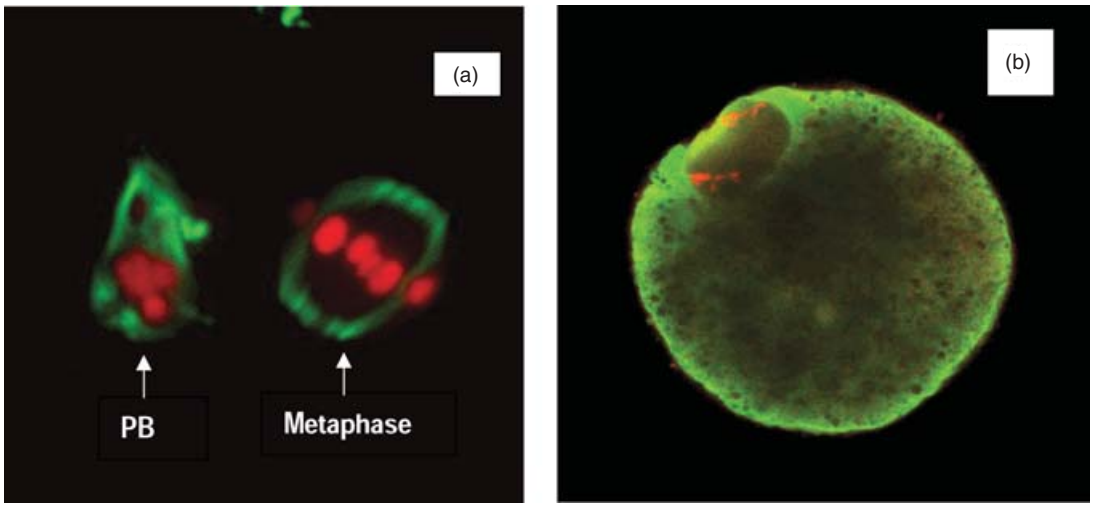

Figure 2 Microtubule distribution in goat oocytes. (a) Meiotic spindle detail in MII adult goat oocyte. DNA appears in red and tubulin in green. PB, polar body. (b) GV stage goat oocyte showing an undefined tubulin network within the cytoplasm.

Table 4 Microtubule organization according to nuclear stage of adult goat oocytes at 0 and 27 h after *in vitro* maturation

			Microtubule organization							
Nuclear stage	*n* (%)		UN *n* (%)		S *n* (%)		S + PB *n* (%)		PB *n* (%)	
0 h of IVM ($n = 30$)										
GV	20	(66.7)	20	(100.0)	–	–	–	–	–	–
MII	10	(33.3)	–	–	3	(30.0)	6	(60.0)	1	(10.0)
27 h of IVM ($n = 29$)										
GV	2	(6.9)	2	(100.0)	–	–	–	–	–	–
MI	5	(17.2)	–	–	5	(100.0)	–	–	–	–
MII	22	(75.9)	–	–	–	–	22	(100.0)	–	–

GV, germinal vesicle; MI, metaphase I; MII, metaphase II; UN, undefined structure; S, meiotic spindle; S + PB, meiotic spindle and polar body; PB, polar body.

Table 5 Microtubule organization according to nuclear stage of prepubertal goat oocytes at 0 and 27 h after *in vitro* maturation

			Microtubule organization							
Nuclear stage	*n* (%)		UN *n* (%)		S *n* (%)		S + PB *n* (%)		PB *n* (%)	
0 h of IVM ($n = 107$)										
GV	81	(75.7)	81	(100)	–	–	–	–	–	–
MI	26	(24.3)	2	(7.7)	24	(92.3)	–	–	–	–
27 h of IVM ($n = 100$)										
GV	7	(7.0)	7	(100)	–	–	–	–	–	–
MI	12	(12.0)	3	(25.0)	9	(75.0)	–	–	–	–
MII	81	(81.0)	4	(4.9)	17	(21.0)	58	(71.6)	2	(2.5)

GV, germinal vesicle; MI, metaphase I; MII, metaphase II; UN, undefined structure; S, meiotic spindle; S + PB, meiotic spindle and polar body; PB, polar body.

symmetrical, barrel-shaped, located at the periphery and oriented tangentially to the cell surface.

Table 5 presents the results of the analysis of microtubule organization of prepubertal goat oocytes. As was observed in adult goat oocytes, an undefined microtubule network was shown in 100% of GV oocytes (Fig. 2b). However, after 27 h of IVM, a significantly ($p < 0.0199$) lower percentage (71.6%) of oocytes at MII

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Table 6 Microtubule organization according to nuclear stage of prepubertal goat IVM-IVF oocytes at 20 h post-insemination ($n = 91$)

Nuclear stage	*n* (%)	Microtubule organization									
		UN *n* (%)		PN *n* (%)		S + PB *n* (%)		PB *n* (%)		Z *n* (%)	
GV	5 (5.5)	5	(100)	–	–	–	–	–	–	–	–
MII	6 (6.6)	–	–	–	–	4	(66.6)	2	(33.3)	–	–
1PN	1 (1.1)	–	–	1	(100)	–	–	–	–	–	–
1PN+Z	6 (6.6)	4	(66.6)	–	–	–	–	–	–	2	(33.3)
2PNs	31 (34.0)	23	(74.2)	3	(9.6)	–	–	5	(16.2)	–	–
2PNa	13 (14.3)	10	(76.9)	1	(7.7)	–	–	–	–	2	(15.4)
PS	22 (24.2)	12	(54.5)	–	–	–	–	–	–	10	(45.4)
Cells	7 (7.7)	7	(100.0)	–	–	–	–	–	–	–	–

GV, germinal vesicle; MII, metaphase II; 1PN, one pronucleus; 1PN+Z, one pronucleus and one sperm head; 2PNs, two synchronous pronuclei; 2PNa, two asynchronous pronuclei; PS, polyspermic; Cells, two or more cells; UN, undefined structure; PN, pronuclei; S + PB, meiotic spindle and polar body; PB, polar body; Z, sperm head.

showed microtubules at the second meiotic spindle and within the polar body, compared with adult goat oocytes.

Microtubule distribution in zygotes of IVM-IVF prepubertal goat oocytes

Table 6 presents the results of the analysis of microtubule distribution in 91 prepubertal *in vitro* matured oocytes at 20 h post-fertilization. The percentage of fertilization was 86.8% (79/91), and 34% (31/91) showed two synchronous pronuclei (2PN). An undefined microtubular network was shown in 74.2% of synchronic 2PN zygotes and in 76.9% of 2PN asynchronous zygotes. Some microtubule polymerization was seen around sperm heads in 45.4% of the polyspermic zygotes.

Discussion

In the present study, a description of microfilament and microtubule organization of prepubertal goat oocytes before IVM (0 h), after IVM (460 oocytes) and 20 h after IVF (184 oocytes) is provided. Moreover, a comparative description of 118 oocytes of adult goats before and after IVM is reported.

Global data from our study (microfilament and microtubule oocytes studied) show that at the onset of culture for maturation 31% (18/57) of the oocytes from adult goats and 41% of the oocytes from prepubertal goats had reinitiated meiosis at the time of oocyte collection from follicles. This high percentage of abnormally matured oocytes could be due to the heterogeneous healthy and atretic follicles from which the oocytes were harvested and, consequently, could be one of the causes of the low embryo development observed in our previous studies (Izquierdo *et al.*, 1999; Rodriguez-González *et al.*, 2003).

Microfilament distribution study

In different species (mouse, rat, pig, human and horse), during oocyte maturation, microfilament organization changes from an even distribution throughout the ooplasm during the GV stage to a concentration in the cortical region and subsequent development of secondary rich domains over the meiotic spindle and around the polar body. In our study, before IVM, microfilament distribution in GV oocytes from prepubertal goats was similar to that in oocytes from adult goats. In both types of oocytes, the microfilaments presented a cortical distribution throughout the oocyte. Also, in bovine oocytes at the GV stage, microfilaments were seen in the cortex (Kim *et al.*, 2000). After 27 h of IVM, microfilament distribution was shown to be different in MII in prepubertal and adult goat oocytes. In adult goat oocytes, an actin-rich domain that was not observed in MII prepubertal goat oocytes overlies the MII spindle. Microfilaments within the polar body were not observed in the majority of the MII prepubertal goat oocytes but were observed in oocytes from adult goats. The lack of actin microfilaments within the polar body could be related to a lack of cytoplasmic actin or a lack of actin polymerization. Our results on adult goat oocytes agree with those of Kim *et al.* (2000), who observed a thick microfilament organization overlying the metaphase plate as well as microfilament furrows between chromatin during polar body extrusion in bovine oocytes. These authors stated that microfilaments were involved in the peripheral location of chromosomes and were also responsible for inducing polar body extrusion. Similar microfilament organization has been observed in the mouse (Longo & Chen, 1985). It has been hypothesized that actin filament structures at the site of the second meiotic spindle and polar body extrusion may play a role in metaphase spindle rotation (Maro *et al.*,

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1986) and in the repair of the cytoplasmic membrane (Terada *et al.*, 2000). As in the mouse, the metaphase spindle in goat oocytes is located tangentially to the plasma membrane and its rotation is required before polar body extrusion. Webb *et al.* (1986) reported that this thick, cortical microfilament structure near the metaphase plate disappeared during the ageing of mouse oocytes. In such conditions, the metaphase plate migrated towards the centre of the egg and this may be the major cause of formation of two female pronuclei without polar body extrusion. In our study, prepubertal goat oocytes did not show a thick, cortical microfilament structure; however, the metaphase spindle was located peripherally. It has been postulated that culture conditions (temperature, pH and ionic stress) could affect microfilament polymerization (Wang *et al.*, 1999). Yang *et al.* (1998) found higher levels of reactive oxygen species (ROS) in fragmented human embryos than in normal embryos. It is known that ROS increase membrane permeability and cause cell damage (Aitken *et al.*, 1989) and DNA fragmentation (Halliwell & Aruoma, 1991) and may also inhibit or delay polymerization and depolymerization of actin filaments resulting in a nucleus–cytoplasm division synchronically causing cell fragmentation or binucleation. Microtubules in human meiotic spindles are highly sensitive to temperature variation (Sathananthan *et al.*, 1988) and some environmental perturbances (Pickering *et al.*, 1990) that can affect genetic balance resulting in chromosomally abnormal cells (Munné *et al.*, 1994). In our study, culture conditions were similar for adult and prepubertal goat oocytes, and thus this difference in microfilament distribution during *in vitro* maturation is more correlated with the female donor's age than with culture conditions. After IVF, most of the sperm-penetrated oocytes showed a microfilament distribution in the cortex in spite of monospermic or polyspermic penetration and pronuclear development. Actin filaments around spermatozoa heads were observed in only 4 oocytes and microfilaments around pronuclei were observed in only 8 oocytes. It has been postulated that actin microfilaments do not participate in pronuclear formation but do participate in pronuclear migration and apposition (Kim *et al.*, 1997*b*). Thus, microfilaments tend to organize around both pronuclei during pronuclear apposition (Kim *et al.*, 1997*a*; Wang *et al.*, 1999). Of the prepubertal goat zygotes with two synchronous pronuclei only 12% displayed microfilaments around both pronuclei. We do not know whether this nearly permanent cortical microfilament distribution observed in prepubertal goat oocytes at a different nuclear stage before and after sperm penetration may be due to: (a) a lower amount of actin produced by the oocyte from prepubertal goats, (2) reduced dynamic actin polymerization or (3) a different actin distribution. The different microfilament distribution observed after IVM could lead to the abnormal distribution after fertilization and, consequently, could be one of the causes for the low incidence of embryo development of prepubertal goat oocytes observed in our previous studies (Izquierdo *et al.*, 1999; Rodriguez-Gonzalez *et al.*, 2003). Moreover, it has been observed that microfilaments are important for normal mitochondrial distribution (Barnett *et al.*, 1997), mitochondrial transfer to daughter cells during cell cleavage (Boldogh *et al.*, 1998) and mRNA distribution (Bassell *et al.*, 1994), all of which could affect cellular cleavage directly or indirectly (Matsumoto *et al.*, 1998).

It is important to mention the presence of strands of microfilament material across the zona pellucida that comes from cumulus cells and contacts oocytes (prepubertal and adult *in vitro* matured oocytes). Although it was not quantified, a similar distribution between prepubertal and adult goat oocytes was observed.

In summary, microfilament distribution in prepubertal goat oocytes presents anomalies in GV, MII and zygotes. Velilla *et al.* (2004) also observed a different cortical granule (CG) distribution in zygotes compared with adult and prepubertal goats. It is well known in mouse and hamster oocytes that an intact microfilament network is required to achieve CG exocytosis (Tahara *et al.*, 1996; Dimaggio *et al.*, 1997). Adult goat fertilized oocytes that have undergone the cortical reaction are characterized by clusters of CGs distributed through the oocyte cortex that are not present in prepubertal goat oocytes (Velilla *et al.*, 2004). Some studies have shown that actin filaments (Sun *et al.*, 2001) participate in CG migration during meiotic maturation and also act as a barrier to prevent premature exocytosis of CG inside the perivitelline space (Nakata & Hirowaka, 1992). Hence, the lack of actin filaments observed could be responsible, at least in part, for the different cortical reaction observed in prepubertal goat oocytes (Velilla *et al.*, 2004).

Microtubule distribution study

Prepubertal and adult oocytes at the GV stage have an undefined microtubule organization. A similar pattern was observed in pig oocytes (Kim *et al.*, 1996*a*), where microtubules were not detected at all at the GV stage. In mouse (Messinger & Albertini, 1991) and horse oocytes (Tremoleda *et al.*, 2001) a uniform complex of microtubules is distributed throughout the cytoplasm of GV stage oocytes.

In vitro matured adult goat oocytes at the MII stage have microtubules organized into a meiotic spindle in metaphase oocytes. A furrow of microtubules among chromatin material within the polar body was observed in MII oocytes. The percentage of prepubertal goat

oocytes at MII with this microtubule organization was significantly lower than in adult goat oocytes. A variation in microtubule organization was shown in prepubertal goat oocytes. Five per cent of MII oocytes after 27 h of IVM showed an undefined structure of microtubules indicating a lack of microtubule polymerization and in 21% tubulin was present on the metaphase spindle without being present inside the polar body. In cow MII oocytes, the only microtubules present were those found in the meiotic spindle (Navara *et al.*, 1994), as was observed in pig (Kim *et al.*, 1996*a*,*c*) and horse oocytes (Tremoleda *et al.*, 2001). This tubulin polymerization has been reported in some other species and has been associated with oocyte developmental competence (Kim *et al.*, 1998). In our study, prepubertal and adult goat oocytes at MII showed microtubules concentrated in the meiotic spindle and inside the polar body. As in the mouse (Messinger & Albertini, 1991), pig (Kim *et al.*, 1996*a*), cow (Aman & Parks, 1994), horse (Tremoleda *et al.*, 2001) and human (Pickering *et al.*, 1988) the meiotic spindle in goat oocytes is a symmetrical barrel-shaped structure with two poles. Similar to rodents and in contrast to other ungulates and primates, the goat oocyte metaphase spindle is located tangentially to the plasma membrane. In the mouse, maternal centrosome material determines the organization and shape of the meiotic spindle (Howlett *et al.*, 1985) and several microtubule organizing centers (MTOCs) within the cytoplasm (Maro *et al.*, 1986), which have not been described in other species such as pig (Kim *et al.*, 1996*a*), sheep (Le Guen & Crozet, 1989) and human (Kim *et al.*, 1998). In this study, MTOCs were not been detected in goat oocytes.

In spite of the differences observed between the microtubule organization in prepubertal and adult goat oocytes in this study, there were no differences in the percentages of oocytes that reach complete nuclear maturation.

In most mammalian species polymerized microtubules are observed around both pronuclei (Simerly *et al.*, 1995). In contrast, in our study, an undefined structure of microtubules was observed after fertilization of prepubertal goat oocytes. Also, disorganized tubulin (not organized as an aster) was observed around condensed sperm heads in 1PN, asynchronous 2PN and polyspermic oocytes. These results are similar to those obtained by Kim *et al.* (1996*b*) in polyspermic pig oocytes, where multiple spermatic asters were observed in polyspermic oocytes associated with penetrating spermatozoa.

The pattern of microtubule polymerization and organization observed in IVM-IVF prepubertal goat oocytes may indirectly alter other cellular processes such as mitochondrial migration, meiotic spindle formation and chromosome segregation and may also be responsible, in part, for chromosome abnormalities (Villamediana *et al.*, 2001) and embryonic arrest observed in prepubertal goat oocytes (Izquierdo *et al.*,1999).

In conclusion, after *in vitro* maturation, prepubertal goat oocytes show a different cytoskeletal organization to adult goat oocytes. These differences were mainly a lack of actin thickening over the metaphase II plate and within the polar body in prepubertal goat oocytes. In oocytes at MII, microtubules were concentrated in the meiotic spindle and within the polar body, in both adult and prepubertal goat oocytes, although this pattern was seen at a lower frequency in prepubertal oocytes. After *in vitro* fertilization, zygotes from prepubertal goat oocytes were characterized by microfilament organization around the cortex and not around the pronuclei, as described in most mammalian species. Also, zygotes from prepubertal goat oocytes were characterized by an undefined microtubule structure.

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