Cytoplasmic changes and developmental competence of bovine oocytes cryopreserved without cumulus cells

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The cryopreservation of female gametes is still an open problem because of their structural sensitivity to the cooling-and-freezing process and to the exposure to cryoprotectants. The present work was aimed to study the effect of vitrification on immature bovine oocytes freed of cumulus cell investment before freezing. To verify the feasibility and efficiency of denuded oocyte (DO) cryopreservation, the cytoplasmic alterations eventually induced either by cell removal or by the vitrification process were analyzed. In particular, the migration of cortical granules and Ca^{++} localization were studied. In addition, the localization and distribution of microtubules and microfilaments in immature fresh and vitrified DOs were evaluated. Finally, to establish whether the removal of cumulus cells influenced developmental competence, DOs were thawed after vitrification, matured *in vitro* and fertilized; then presumptive zygotes were cultured to reach the blastocyst stage. The results indicate that mechanical removal of cumulus cells from immature bovine oocytes does not affect their maturation competence but reduces the blastocyst rate when compared with intact cumulus oocyte complexes (COCs). The findings indicate further that the vitrification process induces changes of cytoplasmic components. However, the composition of the manipulation medium used to remove cumulus cells plays a crucial role in reducing the injuries caused by cryopreservation in both cytoplasmic and nuclear compartments. In fact, the presence of serum exerts a sort of protection, significantly improving both oocyte maturation and blastocyst rates. In conclusion, we demonstrate that denuded immature oocytes can be vitrified after cumulus cells removal and successfully develop up, after thawing, to the blastocyst stage, following *in vitro* maturation and fertilization.

Key words: oocyte, vitrification, *in vitro* maturation, cytoskeleton, tubulin, actin, calcium, cortical granules, bovine.

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Cryopreservation of oocytes seems to be the most promising technique for the storage of female germ cells in different mammals, including man. Several studies have demonstrated that bovine oocytes are much more difficult to be cryopreserved than embryos and sperms. Besides its economic relevance, the bovine species is utilized because of the wide knowledge of oocyte biology, and it represents a model for the study of problems related to mammalian assisted reproduction.

The peculiar morphological and functional characteristics of the bovine oocytes, such as their size, the dynamics of cytoplasmic organelles (Hyttel *et al.* 1986), and the active biochemical process, along with the progress of meiosis (Sirard *et al.* 1989), may account for its susceptible nature to cryopreservation.

Different protocols, such as slow (Lim *et al.* 1999; Suzuki *et al.* 1996) rapid (Hochi *et al.* 1998; 2001) or ultrarapid cooling (Martino *et al.* 1996; Vajta *et al.* 1998) have been designed. However, to date, cryopreservation of bovine oocyte is not yet a well-established technology. Recent progress suggests that ultra-rapid cooling such as *vitrification* could be a feasible and successful approach to oocyte cryopreservation (Arav *et al.* 2002; Vajta 2000). In addition to the technique adopted, the meiotic stage of oocytes also affects the survival of mammalian female gamete after cryopreservation. Many specific protocols in fact have been described using oocytes at different stages of maturation.

Mature oocytes have not yet been effectively cryopreserved, due to their structural sensitivity to cooling and freezing and to the exposure to cryoprotectants. The major abnormalities observed are meiotic spindle disorganization or disruption (Chen *et al.* 2003; Rho *et al.* 2002), which causes chromosomal aberrations, increases polyploidy and impairs fertilization (Saunders and Parks 1999). Freezing of immature oocytes might avoid these problems since genetic material is enclosed within the nuclear enve-

lope, but very low survival rates have been reported (Matsumoto *et al.* 2001; Men *et al.* 2002; Vieira *et al.* 2002). This sensitivity is probably due to a relatively low stability of the cell membrane, to the particular cytoskeleton organization, and to the damage or interruption of the trans-zonal projection of cumulus cells (Hochi *et al.* 1996), which control the intercellular communication between cumulus cells and oocytes during the growth phase and final maturation of the antral follicle. Furthermore, oocytes and cumulus cells have different sizes and require different freezing conditions in order to be preserved, since it has been demonstrated that for every cell type, there is an optimum rate of cooling which depends also on the surface-to-volume ratio of the cell (Shaw *et al.* 2000).

Most workers cryopreserve immature oocytes as intact cumulus oocyte complexes (COCs) (Matsumoto *et al.* 2001; Otoi *et al.* 1995; Suzuki *et al.* 1996) or as partially denuded oocytes (Vajta 2000; Vajta *et al.* 1998). However, after thawing, maturation efficiency and developmental rates after fertilization are very low. It has been postulated that cumulus cells and glyocoproteins slow cryoprotectant penetration either at equilibration or at dilution, and this may result in an uneven intracellular distribution of the cryoprotectant (Hyttel *et al.* 2000).

In humans, several studies have focused on the effect of denuding oocytes before cyostorage using slow freezing, but controversial results are reported (Fabbri *et al.* 2001). In bovine, the few data available report only a limited capability of denuded oocytes (DO) to reach the metaphase II (MII) stage after thawing (Im *et al.* 1997).

Starting from these observations, the possibility to cyopreserve immature bovine oocytes freed of cumulus cell investment before vitrification was studied. To verify the feasibility and efficiency of such an approach, we analyzed the possible cytoplasmic alterations induced by either cell removal or cryopreservation, through the localization and distribution of microtubules and microfilaments in immature DOs before or after vitrification, using histochemical and immunocytochemical techniques. Moreover, since it has been demonstrated that cryopreservation induces premature cortical granule migration and exocytosis (Carroll *et al.* 1990), a process involved in the block of polyspermy in mammalian eggs (Sun 2003) and mediated by Ca^{++}, we also evaluated cortical granules behavior and Ca^{++} localization.

To verify the possibility to reduce the negative effects induced by vitrification on DO, different media were tested during cumulus cell removal procedure Finally, to establish whether the removal of cumulus cells influences the oocyte maturation competence, fresh and cryopreserved DOs were matured *in vitro.* To assess their developmental competence following cryopreservation, thawed oocytes were matured, fertilized and presumptive zygotes were cultured to reach the blastocyst stage.

Materials and Methods

Oocyte collection and maturation in vitro

All chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA), unless otherwise stated.

Bovine ovaries were obtained from a local abattoir and transported to the laboratory, within 2 h in sterile saline (9 g of NaCl/l) at 32-34°C.

COCs were retrieved from 2-6 mm follicles with an 18-gauge needle mounted on an aspiration pump (COOK-IVF, Australia) under a vacuum pressure of -28 mm/Hg. The COCs were examined under a stereomicroscope, and only the medium-brown colored ones, with five or more complete layers of cumulus cells and a finely granulated homogenous ooplasm, were used. Selected COCs were then washed twice in M199 additioned with HEPES 20 mM and 0.4% of BSA (HM199). The whole procedure was performed in approximately 30 min.

The basic maturation medium (bMM) was TCM-199 supplemented with 0.68 mM L-glutamine, 0.2 mM sodium pyruvate, 25 mM $NaHCO_3$, 0.4% fatty-acid-free bovine serum albumin, 0.1 IU/ml human recombinant FSH (hrFSH, Gonal-F, Serono, Rome, Italy). Groups of about 30 COCs (controls) were cultured in 500 μL of bMM for 24 h, in four-well dishes (NUNC, VWR International, Milan, Italy) at 38.5°C under 5% CO_2 in humidified air. DOs were co-cultured with intact COCs (Luciano *et al.* 2004), in order to sustain their *in vitro* maturation. Briefly, DOs were obtained by mechanical removal of cumulus cells by vortexing (see experiment 1). Groups of 15 DOs were matured in co-culture with COCs in a 1:1 ratio.

In vitro fertilization and embryo culture

After maturation, oocytes were fertilized as previously described (Luciano *et al.* 1999). Briefly, the

contents of a straw of cryopreserved bull spermatozoa (CIZ, S. Miniato, Pisa, Italy) was thawed and cells separated on a 45-90% Percoll gradient. Sperm was diluted to a final concentration of $0.5x10^6$ spermatozoa/mL in fertilization medium (TALP supplemented with 0.6% w/v of fatty-acid-free BSA, 10 μg/mL heparin, 20 μM penicillamine, 1 μM epinephrine, and 100 μM hypotaurine. Groups of about 30 COCs were cultured in 300 μL of fertilization medium and incubated for 18 hours, in four-well dishes, at 38.5° C in 5% CO_2 in humidified air. DOs matured in co-culture during IVM were separated from intact COCs by using a narrow-bore pipette, before IVF.

Embryo culture medium was a modified synthetic oviduct fluid (SOF), (Tervit *et al.* 1972), buffered with 25 mM $NaHCO_3$, supplemented with MEM essential and non-essential amino acids, 0.72 mM sodium pyruvate, 2.74 mM myo-inositol, 0.34 mM sodium citrate and 5% calf serum (SOF-C). Embryo rinse and manipulation medium was SOF supplemented with 0.3% (w/v) BSA fraction V, MEM essential and non-essential amino acids, 0.72 mM sodium pyruvate and buffered with 10 mM HEPES and 5 mM $NaHCO_3$ (SOF-R).

After fertilization, presumptive zygotes were vortexed for 2 min in 500 μL SOF-R, rinsed twice and then transferred in groups of about 30 in 400 μL of SOF-C under 400 μl of mineral oil. Incubation was performed in a modified atmosphere with 5% CO_2, 5% O_2 and 90% N_2. Cleavage and blastocyst rates were respectively assessed after 48 h and 186 h post insemination. At the end of culture period, blastocysts were fixed in 60% methanol in PBS and cell nuclei were counted under fluorescence microscopy after staining with 0.5 mg/mL propidium iodide.

Oocyte vitrification

Oocyte vitrification was carried out following the method of open pulled straws (OPS, Minitube, Tiefenbach-Germany) (Vajta *et al.* 1998). Briefly, groups of 3-5 DOs were initially equilibrated for 1 min in holding medium (HM) consisting of 20 mM Hepes-buffered TCM-199 supplemented with 20% calf serum. After equilibration, oocytes were incubated in 10% ethylene glycol (EG) and 10% dimethyl sulfoxide (DMSO) dissolved in HM for 30 sec and successively transferred into 20% EG and 20% DMSO and 1 M sucrose dissolved in HM for 25 sec. Oocytes were loaded in straws and immediately submerged into liquid nitrogen. Warming was performed by placing the end of the straw directly into the HM supplemented with 0.3 M sucrose. The vitrified medium became liquid within 1-2 seconds, whereupon the HM entered the straw. The oocytes flowed out of the straw into the HM where they remained for 1 min. Successively, oocytes were transferred into and washed for 5 min in 0.2 M sucrose supplemented HM, then washed in HM and processed according to the experimental design.

Immunolocalization of microfilaments and microtubules

Cytoskeleton organization and nuclear maturation of oocytes were examined by three-color fluorescence staining. Immunolocalization of tubulin was performed following Wu *et al.* (1999). Briefly, vitrified and fresh DOs were fixed in 0.3% Triton X-100 and 2% paraformaldehyde in PBS at 30° for 1 hr. After an incubation of 10 min at room temperature in PBS containing 1% BSA, 1% NDS (Normal Donkey Serum) and 0.3% Triton-X100, the oocytes were incubated overnight at 4°C with an anti-α-tubulin monoclonal antibody diluted 1:200 in PBS, and then with a fluorescein isothiocyanate (FITC) coniugated anti-mouse IgG antibody raised in donkey (Jackson Immunoresearch lab, Inc. West Grove, PA) for 1 hr at room temperature. To detect the distribution of microfilaments, the oocytes were then exposed to tetramethylrhodamine B isothiocyanate (TRITC) labelled phalloidin (1 μg/ml for 30 min at 37°C). Chromatin DNA was stained with DAPI (1 μg/mL in PBS; Molecular Probes Europe BV, Leiden, The Netherlands).

All stained samples were mounted with an anti-fade medium (Vectashield Vector Lab., Burlingame, CA, USA). As control, some DOs were treated with unlabeled phalloidin before incubation with labeled filaments marker and incubated directly with the secondary antibody, without pre-treatment with primary anti-tubulin antibody. All samples were then mounted with an anti-fade medium and observed with a conventional epifluorescence microscope (Nikon, Eclipse E 600,) or using a confocal laser scanning microscope (Leica DCNT, Heidelberg, Germany) equipped with a krypton-argon and a helium-neon laser for UV.

Morphological evaluation

Some oocytes from each experimental group were fixed in 2.5% glutaraldehyde in PBS (pH 7.4) for

4 hr at 4°C and post fixed in 0.1M sodium phosphate buffer containing 1% OsO_4 for 1 hr at 4°C. Finally individual oocytes were dehydrated by ethanol and embedded in Epon resin. Semi-thin sections (2 μm) were stained with 0.5% toluidine blue for light microscopy observations.

Cortical granules localization

To see the localization and distribution of cortical granules (CGs), oocytes were processed according to Pocar *et al.* (2001). Briefly, after cumulus cells removal, oocytes were treated with pronase E (0.5% w/v) pre-warmed at 39°C to remove the zona pellucida. Dezoned oocytes were washed twice in SOF. After rinses with 10% fetal calf serum (FCS) diluted in PBS, oocytes were fixed in 3% paraformaldehyde in PBS for 45 min at room temperature and incubated in a blocking solution for 30 min (1% BSA, 0.1 M glycine and 0.05% triton X100 in DPBS). Finally, samples were exposed for 30 min at room temperature to 10 μg/mL FITC-conjugated *Lens culinaris* agglutinin diluted in the blocking solution, in the dark. Labelled cells were washed three times in BS for 10 minutes. To assess the nuclear maturation stage, samples were stained with DAPI as previously described.

Oocytes were classified into 3 classes according to the pattern of distribution of the CGs following Izadyar *et al.* (1998): type I (CG1): large aggregates, localized throughout the cytoplasm; type II (CG2): localized in the cortical cytoplasm and distributed as individual particles or small aggregates; type III (CG3): evenly dispersed as single particles in the cortical cytoplasm along the oolemma.

Calcium localization

To localize intracellular calcium, DOs were incubated for 30 min at room temperature with a 10 μM solution of Fluo-3 (Molecular Probes OR, USA) dissolved in DMSO (Luciano *et al.* 1994). After dye loading, oocytes were observed under a fluorescence microscope and the percentages of oocytes with punctuate or diffuse pattern of free intracellular calcium distribution were estimated.

Statistical analysis

All experiments were repeated at least three times. Maturation, cleavage and blastocyst rates were calculated by ANOVA followed by Fisher protected LSD test. The differences in the distribution pattern of CGs and of actin microfilaments were calculated by the χ^2 test. Differences of $p<0.05$ were considered as significant.

Experimental design

Experiment 1: Effect of cumulus cells removal on meiotic competence of fresh and vitrified denuded oocytes

In order to assess whether the medium composition could affect oocyte viability during the removal of cumulus cells, two media, which are commonly employed during the manipulation of female gametes were used to remove cumulus investment. After selection, COCs were freed of cumulus cells by vortexing for 2 min at 35 Htz using 1) PBS supplemented with 0.1% polyvinyl alcohol (PBS/PVA) or 2) M199 supplemented with 5% calf serum and buffered with 20 mM HEPES (M199HCS).

DOs were then vitrified as mentioned above. After thawing, DOs were matured for 24 hr as described above. As controls, groups of fresh DOs, denuded under the same conditions, and a group of intact COCs were matured *in vitro.* At the end of culture, oocytes were fixed in 60% methanol in PBS and the nuclear stage of meiotic progression was assessed by fluorescence microscopy after staining with 0.5 mg/mL propidium iodide.

Experiment 2. Cytoplasmic changes related to the cryopreservation of denuded oocytes

The second experiment was designed to evaluate cytoplasmic changes eventually induced by cumulus cells removal before and after cryopreservation of DOs. In particular, the distribution of cortical granules, the organization of microtubules and microfilaments and oocyte morphology were analyzed as parameters of cytoplasmic quality, before and after maturation both in fresh and frozen/thawed DOs. In addition, the Ca^{++} localization was analyzed in immature oocytes, before and after vitrification.

Starting from the results obtained in experiment 1, oocytes were denuded either with PBS/PVA or M199HCS in order to analyze also a possible correlation with the type of medium used in the cumulus cells removal procedure.

Experiment #3. Developmental capability of denuded oocyte following vitrification and thawing

Fresh and vitrified-thawed DOs were matured *in vitro* and fertilized, and the presumptive zygotes were cultured for 7 days to evaluate the blastocyst

rate as a parameter of their developmental capability *in vitro.*

Results

Effect of cumulus cells removal on meiotic competence of fresh and vitrified DOs

No differences were observed in the maturation rate between fresh DOs (after either PBS/PVA or M199HCS) and the control group (83.9±3.5, 86.1±2.1 and 89.7± 2.9, $p<0.05$, respectively). The maturation capability of oocytes after vitrification was significantly affected by the type of medium used to remove cumulus cells (Table 1). The use of M199HCS significantly improved the oocyte competence to reach the meiotic stage of metaphase II, similarly to intact COCs.

Cytoplasmic changes related to the cryopreservation of DOs

As indicated in Table 2, denuded immature oocytes showed only CG1 and CG2 patterns (Figure 1 a, b), both in fresh and vitrified samples. Oocytes denuded either with PBS/PVA or M199HCS did not show significant differences. The number of CG1 was significantly higher in vitrified oocytes denuded with M199HCS than in those denuded in PBS/PVA (24 vs. 10.8, $p<0.05$).

Table 3 shows that after 24h of *in vitro* maturation, CG1, CG2 and CG3 patterns (Figure 1, a, b, c), were present. Fresh DOs mainly showed a CG2 pattern while vitrification induced an increase of CG3. No significant differences were observed in the distribution of the three patterns after either PBS/PVA or M199HCS treatment.

All fresh immature DOs showed calcium deposits localized into cytoplasmic foci (punctuate aspect, Figure 2a). After vitrification, about 60% of the oocytes denuded with M199HCS and 40% of those denuded with PBS/PVA showed the same punctuate pattern; all the remaining oocytes had a diffuse pattern of cytoplasmic calcium distribution (Figure 2b).

No differences in localization and distribution of microtubules were observed in immature oocytes. All the samples displayed a complex network of weakly labeled microtubules distributed uniformly throughout the ooplasm (Figure 3 a, d).

Before vitrification, DOs showed microfilaments homogeneously distributed throughout the ooplasm (Figure 3b), with a slightly greater concentration just beneath the plasma membrane, in a percentage

Table 1. Effect of removal medium on the nuclear maturation competence of vitrified DOs after thawing.

Treatments	*n*	*GV*	*MI*	*MII*	*DEG*
PBS/PVA	80	1.5 ± 1.5	38.5 ± 8.9 [b]	42.9 ± 5.8 [a]	17.1 ± 5.1 [b]
M199/CS	73	0	15.3 ± 6.4 [a]	79.3 ± 7.3 [b]	5.3 ± 4.1 [a]

GV, immature germinal vesicle stage; MI, metaphase I; MII mature metaphase II; DEG, degenerated. n= number of oocytes. Values are expressed as mean ± SD. [a, b] value within columns with different superscripts are significantly different (P<0.05).

Table 2. Effect of the cumulus cells removal medium on the localization of CGs in immature oocytes, after thawing

Treatments	*n*	*CG1 (%)*	*CG2 (%)*
PBS/PVA fresh	36	14 (38.8) [bc]	22 (62.2) [ab]
PBS/PVA vitrified	37	4 (10.8) [a]	33 (89.2) [c]
M199/CS fresh	32	20 (62.5) [c]	12 (37.5) [a]
M199/CS vitrified	50	12 (24) [b]	38 (76) [bc]

n= number of oocytes; [a, b, c,] values within columns with different superscripts are significantly different (p<0.05).

Table 3. Effect of the cumulus cells removal medium on the localization of CGs after thawing in oocytes matured *In vitro* for 24 h.

Treatments	*n*	*CG1 (%)*	*CG2 (%)*	*CG3 (%)*
PBS/PVA fresh	28	5 (17.9) [b]	16 (57.1) [b]	7 (25.0) [a]
PBS /PVA vitrified	25	0 (0) [a]	8 (32.0) [a]	17 (68.0) [b]
M199/CS fresh	31	6 (19.4) [b]	19 (61.3) [b]	6 (19.4) [a]
M199/CS vitrified	37	4 (11.4) [b]	14 (40.0) [a]	17 (48.6) [b]

n, number of oocytes. [a, b, c,] values within columns with different superscripts are significantly different (p<0.05).

significantly higher in oocytes denuded with M199HCS than in those denuded in PBS/PVA (59.4 vs. 35.7, $p<0.05$). The process of vitrification did not affect such a distribution (57.1 vs. 47, $p<0.05$).

An aggregation of microfilaments in small clusters scattered throughout the ooplasm was frequently observed in PBS/PVA DOs (Figure 3e).

All oocytes that reached the metaphase II stage of meiotic division (Table 1) showed the same localization and distribution of microtubules and microfilaments. Microfilaments were localized mainly in the cortex, overlying the metaphase chromatin and the polar body (Figure 4a). Microtubules were detected both in the spindle and in the polar body (Figure 4b). Oocytes that did not correctly reach

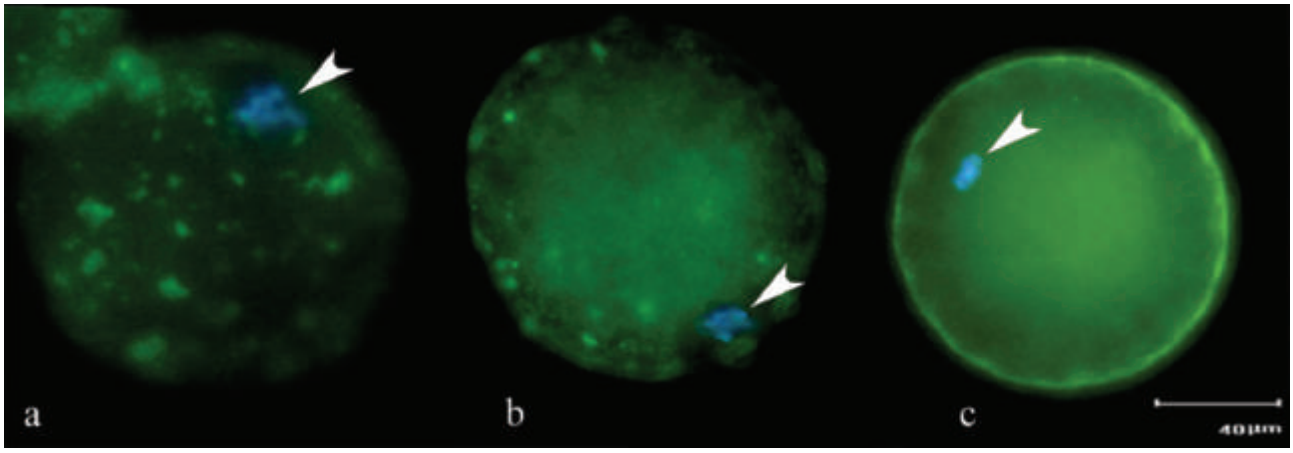

Figure 1. Representative cortical granules distribution patterns in bovine oocytes a) CG1 pattern, where CGs are diffused as large aggregates throughout the ooplasm; b) CG2 pattern, where CGs are distributed as small aggregates and single particles at the periphery of the ooplasm; c) CG3 pattern, where CGs are disposed individually along the oolemma. Chromatin DNA (arrowhead) is stained blue with DAPI. a and b: oocytes at the germinal vesicle stage; c: metaphase II oocyte.

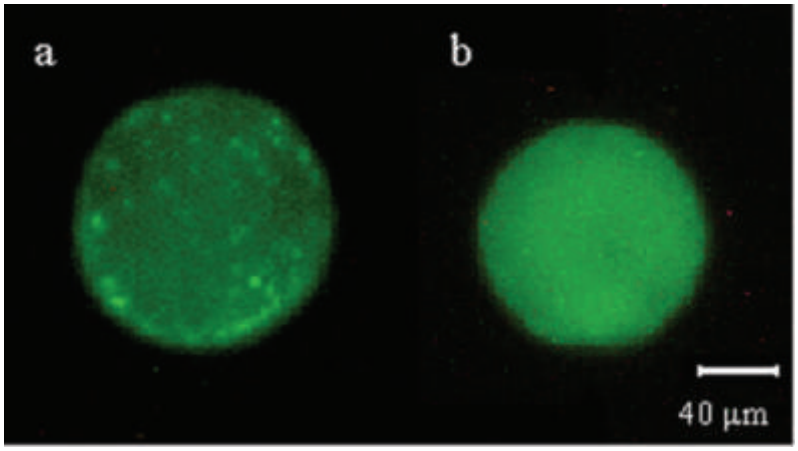

Figure 2. Calcium distribution in immature oocytes a) oocyte with calcium deposits localized in cytoplasmic foci (punctuate pattern); b) oocyte with calcium dispersed in the ooplasm (diffuse pattern).

methaphase II showed tubulin and actin diffuse in the ooplasm, sometimes organized in clusters (Figure 4 d, e).

The morphological observation of semi-thin sections showed that all immature oocytes were characterized by an eccentrically-located nucleus with a well defined, regular envelope (Figure 5). Fresh DOs showed a peripheral distribution of organelles and lipid inclusion and a large number of vacuoles uniformly distributed in the ooplasm; a perivitelline space was detectable (Figure 5a).

After vitrification, the majority of the oocytes denuded in M199HCS showed the same morphological characteristics described above except for an irregular nuclear envelope (Figure 5 b). The majority of oocytes denuded in PBS/PVA were characterized by the presence of clusters of organelles and a great number of vacuoles of different size (Figure 5 c). In some cases, vitrification induced an undulation of oolemma with a consequent enlargement of the perivitelline space (Figure 5b, c). After maturation, all oocytes that reached metaphase II stage did not show significant morphological differences either before or after vitrification (Not Shown).

Developmental capability of DOs after freezing

In Table 4 are reported the results of embryo culture *in vitro* up to the blastocyst stage (day 7). A lower percentage of fresh denuded oocytes reached the blastocyst stage in comparison with intact COCs (23.9 vs. 35.4, $p<0.05$).

After vitrification, only a small percentage of oocytes denuded in M199HCS were able to develop up to the blastocyst stage (4.3%). On the contrary, no blastocysts were obtained from oocytes denuded in PBS/PVA.

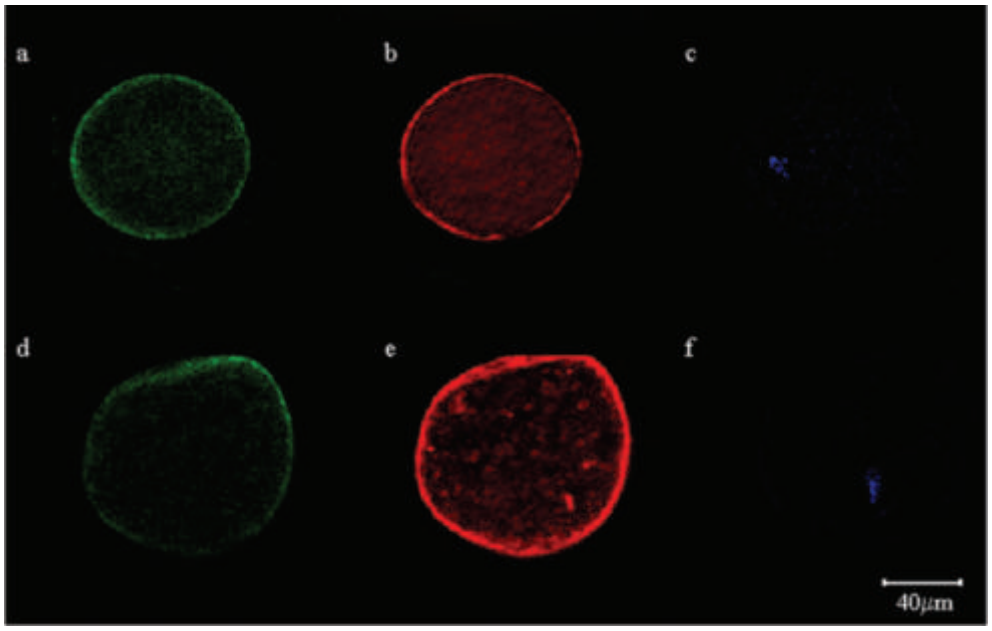

Figure 3. Representative confocal images of immature DOs showing microtubules in green, microfilaments in red, and DNA in blue. a, d) microtubules are uniformly distributed throughout the ooplasm; b) microfilaments diffused in the ooplasm with a slightly greater concentration beneath the oolemma; e) microfilaments diffused irregularly in the ooplasm and partially condensed in small clusters; c, f) germinal vesicle stage chromatin.

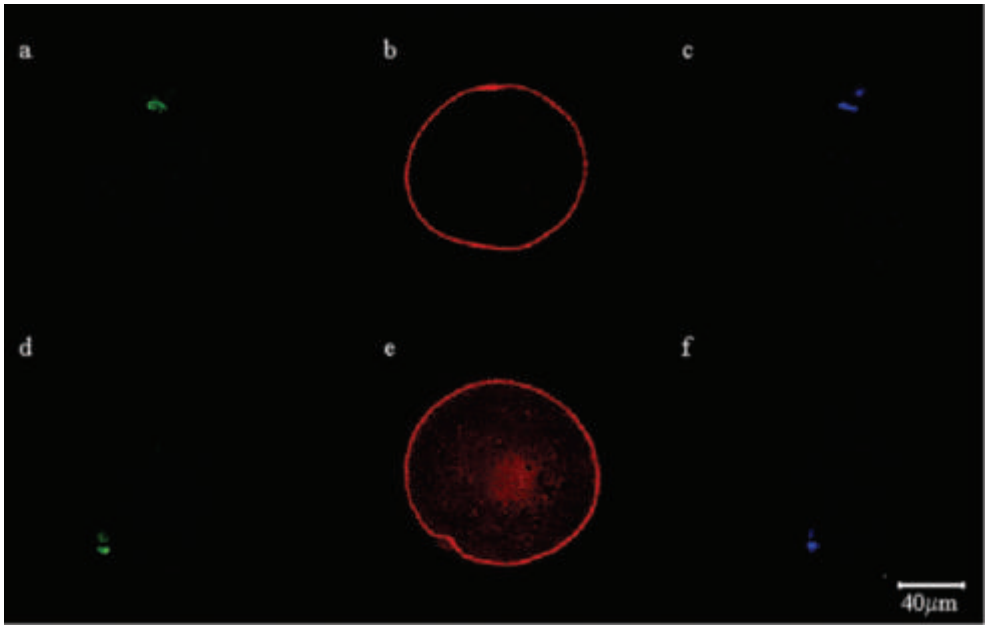

Figure 4. Representative confocal images of DOs after *In vitro* maturation, showing microtubules in green, microfilaments in red, and DNA in blue. a, d) microtubules localized in the spindle and in the polar body; b) microfilaments disposed beneath the oolemma, overlying the metaphase chromatin and the polar body; e) microfilaments disposed beneath the oolemma and diffused in the ooplasm, small aggregates are also present; c, f) chromatin condensed on the metaphase II plate and inside the polar body.

Discussion

Our data demonstrate that during the removal of cumulus cells from immature bovine oocytes, the use of a serum-enriched medium significantly reduces the damage caused by vitrification in both the cytoplasmic and nuclear compartments. The removal of cumulus cells using such a medium preserves the capability of female gametes to reach metaphase II both in fresh and in vitrified oocytes.

It is widely accepted that the morphological and functional modifications observed in the cytoplasm during maturation are directly involved in the acquisition of developmental competence of mammalian oocytes. A common feature of ooplasmic maturation is the redistribution of organelles such as CGs, following resumption of meiosis. CGs, are membrane-

Table 4. Effect of the cumulus cells removal medium on developmental competence after thawing

Treatment	*n*	*% Cleaved*	*% Blastocyst on cleaved*	*% Blastocyst on total*
DOs vitrified (PBS/PVA)	78	30.1±1.8	0	0
DOs vitrified (M199HCS)	71	75.9±1.6 [a]	5.7±0.7 [a]	4.3±0.5 [a]
DOs fresh	80	92.6 ±1.9[b]	25.7±2.9 [b]	23.9±3.1 [b]
COCs fresh	74	94.7±1,2[b]	37.4±2.8 [c]	35.4±2.9 [c]

n= number of oocytes; values are expressed as mean ± SE; [a, b, c] values within columns with different superscripts are significantly different (p<0,05)

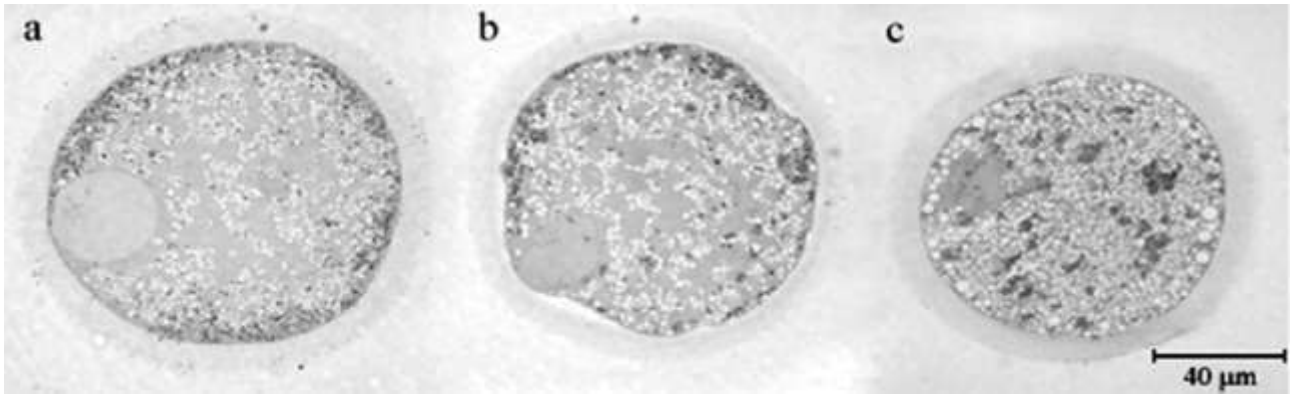

Figure 5. Semi-thin sections of immature fresh (a) or vitrified (b, c) DOs.

bound secretory granules that undergo exocytosis after sperm penetration and are responsible for modifying the extracellular environment (Ducibella 1998) to prevent polyspermy. Therefore, CGs distribution may be considered as a useful marker to evaluate developmental competence (Damiani *et al.* 1996). In bovine oocytes, three distribution patterns of CGs have been identified (Izadyar *et al.* 1998) suggesting that a relationship apparently exists between nuclear stages of maturation and CGs localization. Our data are in agreement with such observations. However, in the present study we show that cumulus cells removal induces a premature redistribution of CGs, and M199HCS medium reduces the incidence of this process, but only in fresh immature oocytes. Vitrification, in fact, induces a significant increase of the CG2 pattern, irrespective of the medium (either M199HCS or PBS/PVA) used to denude the oocytes. An increase of more peripheral CGs pattern has been observed also at the end of maturation of vitrified oocytes suggesting that, following cell removal, the mechanical damage of the cytoskeleton may affect the oocyte integrity. It has been demonstrated, in fact, that microfilaments are anchored to the corona cells through junctional contacts via zona pellucida (Assey *et al.* 1994) and that the translocation of CGs requires a Ca^{++} transient-mediated rearrangement of the cytoskeleton (Raz *et al.* 1998; Sun *et al.* 2001). Ultrastrucural studies performed on mouse and pig demonstrated that in oocytes enclosed in antral follicles, calcium deposits are present within mitochondria, lipid droplets, vacuoles and on the surface of yolk granules, as well as in the caryoplasm (Petr *et al.* 2001; Rozinek *et al.* 2003). A remarkable decrease of Ca^{++} deposits occurs in pig oocytes immediately after isolation from the follicle, probably as a consequence of the manipulation procedures, but during *in vitro* culture Ca^{++} deposits are gradually restored (Petr *et al.* 2001). We have observed that in bovine, oocytes collection and cumulus cells removal does not induce depletion of Ca^{++} deposits, as shown by the punctuate distribution pattern throughout the ooplasm in fresh oocytes. Moreover, following vitrification and thawing, oocyte denudation in the presence of M199HCS often preserves the distribution of calcium deposits in cytoplasmic foci.

Also microfilaments and microtubules play a critical role in the chromosomal and cytoplasmic dynamic events during oocyte maturation and fertilization and are very sensitive to cryopreservation protocols (Saunders and Parks 1999). Studies performed in mouse (Magistrini and Szollosi 1980), human (Park, *et al.* 1997) and bovine (Saunders and Parks 1999) have demonstrated that exposure of the MII oocytes to low temperature, or cryoprotectant resulted in disorganization and loss or clumping of meiotic spindle microtubules; thus freezing at the immature stage may be an alternative approach in the cryopreservation of female gamete since chromatin is uncondensed and no morganized microtubules are present. Tubulin in fact appears evenly distributed in the cytoplasm of pig, mouse (Lee, *et al.* 2000), and horse (Tremoleda, *et al.* 2001) immature oocyte. Also in our experiments we observed a pale signal for tubulin, not affected by cell removal, in both fresh and vitrified bovine Dos. However, in this species, Kim *et al.* (2000) were unable to detect positive signals, probably due to the different method adopted.

In GV stage oocytes microfilaments provide the framework for cell division and are either homogenously distributed in the ooplasm, or immediately close to the oolemma and around the GV. In MII oocytes they are located mainly in the cell cortex overlying the meiotic spindle in pig (Wang, *et al*. 2000), horse (Tremoleda, *et al*. 2001) and cow (Kim, *et al*. 2000). We observed that the use of M199HCS significantly preserves microfilaments distribution, both in unfrozen and frozen oocytes, suggesting that, as previously demonstrated for Ca^{++} and CGs, this medium allows oocytes to be more suitable for cryopreservation.

The use of M199HCS exerts also a favorable effect on the preservation of a correct organelle distribution in the cytoplasm. In fact, in the presence of this medium, the number of oocytes with clustered organelles, and the number of cytoplasmic vesicles was significantly reduced.

Due to the complexity of serum composition, it is still impossible to identify the specific factor(s) protecting oocytes from cryopreservation injuries during removal of the cumulus cells. Further experiments are needed to clarify which macromolecules, hormones and/or growth factors are involved.

In conclusion, our results demonstrate that bovine immature oocytes at the stage of germinal vescicle, after being freed of cumulus cells before vitrification, can be matured and fertilized, and successfully develop *in vitro* to the blastocyst stage, provided that suitable conditions are selected to reduce cytoplasmic damage induced by cumulus cell removal. In particular, the presence of serum in the manipulation medium significantly reduces the negative effects of vitrification.

Although the capability of blastocysts originating from vitrified immature DOs to develop normally up to term has still to be verified, we believe that the blastocyst rate of 4.3% we have obtained after vitrification is only apparently unsatisfactory: in fact, this is the first evidence of successful oocyte cryopreservation for which, to date, only controversial and sporadic data are available for the bovine species (Hochi 2003).

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References

Arav A, Yavin S, Zeron Y, Natan D, Dekel I, Gacitua H. New trends in gamete's cryopreservation. Mol Cell Endocrinol 2002;187:77-81.

Assey RJ, Hyttel P, Greve T, Purwantara B. Oocyte morphology in dominant and subordinate follicles. Mol Reprod Dev 1994;37:335-44.

Carroll J, Depypere H, Matthews CD. Freeze-thaw-induced changes of the zona pellucida explains decreased rates of fertilization in frozen-thawed mouse oocytes. J Reprod Fertil 1990;90:547-53.

Chen SU, Lien YR, Chao KH, Ho HN, Yang YS, Lee TY. Effects of cryopreservation on meiotic spindles of oocytes and its dynamics after thawing: clinical implications in oocyte freezing--a review article. Mol Cell Endocrinol 2003;202: 101-7.

Damiani P, Fissore RA, Cibelli JB, Long CR, Balise JJ, Robl JM, et al. Evaluation of developmental competence, nuclear and ooplasmic maturation of calf oocytes. Mol Reprod Dev 1996;45:521-34.

Ducibella T. Biochemical and cellular insights into the temporal window of normal fertilization. Theriogenology 1998;49:53-65.

Fabbri R, Porcu E, Marsella T, Rocchetta G, Venturoli S, Flamigni C. Human oocyte cryopreservation: new perspectives regarding oocyte survival. Hum Reprod 2001;16:411-6.

Hochi S. Japanese Society for Animal Reproduction: award for outstanding research 2002. Cryopreservation of follicular oocytes and preimplantation embryos in cattle and horses. J Reprod Dev 2003;49:13-21.

Hochi S, Akiyama M, Minagawa G, Kimura K, Hanada A. Effects of cooling and warming rates during vitrification on fertilization of in vitro-matured bovine oocytes. Cryobiology 2001;42:69-73.

Hochi S, Ito K, Hirabayashi M, Ueda M, Kimura K, Hanada A. Effect of nuclear stages during IVM on the survival of vitrified-warmed bovine oocytes. Theriogenology 1998;49:787-96.

Hochi S, Kozawa M, Fujimoto T, Hondo E, Yamada J, Oguri N. In vitro maturation and transmission electron microscopic observation of horse oocytes after vitrification. Cryobiology 1996;33:300-10.

Hyttel P, Vajta G, Callesen H. Vitrification of bovine oocytes with the open pulled straw method: ultrastructural consequences. Mol Reprod Dev 2000;56:80-8.

Hyttel P, Xu KP, Smith S, Greve T. Ultrastructure of in-vitro oocyte maturation in cattle. J Reprod Fertil 1986;78:615-25.

Im KS, Kang JK, Kim HS. Effects of cumulus cells, different cryoprotectants, various maturation stages and preincubation before insemination on developmental capacity of frozen-thawed bovine oocytes. Theriogenology 1997;47:881-91.

Izadyar F, Hage WJ, Colenbrander B, Bevers MM. The promotory effect of growth hormone on the developmental competence of in vitro matured bovine oocytes is due to improved cytoplasmic maturation. Mol Reprod Dev 1998;49:444-53.

Kim NH, Cho SK, Choi SH, Kim EY, Park SP, Lim JH. The distribution and requirements of microtubules and microfilaments in bovine oocytes during in vitro maturation. Zygote 2000;8:25-32.

Lee J, Miyano T, Moor RM. Spindle formation and dynamics of gamma-tubulin and nuclear mitotic apparatus protein distribution during meiosis in pig and mouse oocytes. Biol Reprod 2000;62: 1184-92.

Lim JM, Ko JJ, Hwang WS, Chung HM, Niwa K. Development of in vitro matured bovine oocytes after cryopreservation with different cryoprotectants. Theriogenology 1999;51:1303-10.

Luciano AM, Modina S, Lodde V, Beretta MS, Lauria A. Embryonic development of denuded bovine oocytes matured in the presence of intact cumulus-oocyte complexes. 8th Conference of the European Society of Domestic Animals Reproduction. Warsaw (Poland), 2004:(in press).

Luciano AM, Pappalardo A, Ray C, Peluso JJ. Epidermal growth factor inhibits large granulosa cell apoptosis by stimulating progesterone synthesis and regulating the distribution of intracellular free calcium. Biol Reprod 1994;51:646-54.

Luciano AM, Pocar P, Milanesi E, Modina S, Rieger D, Lauria A, et al. Effect of different levels of intracellular cAMP on the in vitro maturation of cattle oocytes and their subsequent development following in vitro fertilization. Mol Rep Develop 1999;54:86-91.

Magistrini M, Szollosi D. Effects of cold and of isopropyl-n-phenylcarbamate on the second meiotic spindle of mouse oocytes. Eur J Cell Biol 1980;22:699-707.

Martino A, Songsasen N, Leibo SP. Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. Biol Reprod 1996;54:1059-69.

Matsumoto H, Jiang JY, Tanaka T, Sasada H, Sato E. Vitrification of large quantities of immature bovine oocytes using nylon mesh. Cryobiology 2001;42:139-44.

Men H, Monson RL, Rutledge JJ. Effect of meiotic stages and maturation protocols on bovine oocyte's resistance to cryopreservation. Theriogenology 2002;57:1095-103.

Otoi T, Yamamoto K, Koyama N, Suzuki T. In vitro fertilization and development of immature and mature bovine oocytes cryopreserved by ethylene glycol with sucrose. Cryobiology 1995;32:455-60.

Park SE, Son WY, Lee SH, Lee KA, Ko JJ, Cha KY. Chromosome and spindle configurations of human oocytes matured in vitro after cryopreservation at the germinal vesicle stage. Fertil Steril 1997; 68: 920-6.

Petr J, Rozinek J, Hruban V, Jilek F, Sedmikova M, Vanourkova Z, Nemecek Z. Ultrastructural localization of calcium deposits during in vitro culture of pig oocytes. Mol Reprod Dev 2001;58:196-204.

Pocar P, Brevini TA, Perazzoli F, Cillo F, Modina S, Gandolfi F. Cellular and molecular mechanisms mediating the effects of polychlorinated biphenyls on oocyte developmental competence in cattle. Mol Reprod Dev 2001;60:535-41.

Raz T, Ben-Yosef D, Shalgi R. Segregation of the pathways leading to cortical reaction and cell cycle activation in the rat egg. Biol Reprod 1998;58:94-102.

Rho GJ, Kim S, Yoo JG, Balasubramanian S, Lee HJ, Choe SY. Microtubulin configuration and mitochondrial distribution after ultra- rapid cooling of bovine oocytes. Mol Reprod Dev 2002;63: 464-70.

Rozinek J, Vanourkova Z, Sedmikova M, Lanska V, Petr J, Rajmon R, Jilek F. Ultrastructural localisation of calcium deposits in pig oocytes maturing in vitro: effects of verapamil. Zygote 2003;11:253-60.

Saunders KM, Parks JE. Effects of cryopreservation procedures on the cytology and fertilization rate of in vitro-matured bovine oocytes. Biol Reprod 1999;61:178-87.

Shaw JM, Cox SL, Trounson AO, Jenkin G. Evaluation of the long-term function of cryopreserved ovarian grafts in the mouse, implications for human applications. Mol Cell Endocrinol 2000;161:103-10.

Sirard MA, Florman HM, Leibfried-Rutledge ML, Barnes FL, Sims ML, First NL. Timing of nuclear progression and protein synthesis necessary for meiotic maturation of bovine oocytes. Biol Reprod 1989;40:1257-63.

Sun QY. Cellular and molecular mechanisms leading to cortical reaction and polyspermy block in mammalian eggs. Microsc Res Tech 2003;61:342-8.

Sun QY, Lai L, Park KW, Kuhholzer B, Prather RS, Schatten H. Dynamic events are differently mediated by microfilaments, microtubules, and mitogen-activated protein kinase during porcine oocyte maturation and fertilization in vitro. Biol Reprod 2001;64:879-89.

Suzuki T, Boediono A, Takagi M, Saha S, Sumantri C. Fertilization and development of frozen-thawed germinal vesicle bovine oocytes by a one-step dilution method in vitro. Cryobiology 1996;33:515-24.

Tervit HR, Whittingham DG, Rowson LEA. Successful culture in vitro of sheep and cattle ova. J Reprod Fertil 1972;30:493-97.

Tremoleda JL, Schoevers EJ, Stout TA, Colenbrander B, Bevers MM. Organisation of the cytoskeleton during in vitro maturation of horse oocytes. Mol Reprod Dev 2001;60:260-9.

Vajta G. Vitrification of the oocytes and embryos of domestic animals. Anim Reprod Sci 2000;60-61:357-64.

Vajta G, Holm P, Kuwayama M, Booth PJ, Jacobsen H, Greve T, Callesen H. Open pulled straw (OPS) vitrification - a new way to reduce cryoinjuries of bovine ova and embryos. Mol Reprod Dev 1998;51:53-8.

Vieira AD, Mezzalira A, Barbieri DP, Lehmkuhl RC, Rubin MI, Vajta G. Calves born after open pulled straw vitrification of immature bovine oocytes. Cryobiology 2002;45:91-4.

Wang WH, Abeydeera LR, Prather RS, Day BN. Polymerization of nonfilamentous actin into microfilaments is an important process for porcine oocyte maturation and early embryo development. Biol Reprod 2000;62:1177-83.

Wu B, Tong J, Leibo SP. Effects of cooling germinal vesicle-stage bovine oocytes on meiotic spindle formation following in vitro maturation. Mol Reprod Dev 1999;54:388-95.