―**Original**―

Change of the Mitochondrial Distribution in Mouse Ooplasm During In Vitro Maturation

Yayoi Nishi[1], Toshiyuki Takeshita[1], Kahei Sato[2] and Tsutomu Araki[1]

[1]Department of Obstetrics and Gynecology, Nippon Medical School
[2]Department of Applied Biological Science, Nihon University College of Bioresource Sciences, Fujisawa

Abstract

　Mitochondria (mt) have been reported to be closely related to the maturation of mammalian oocytes, but their function in oocyte maturation has not been elucidated. In this study, we examined the kinetics of mt and chromatin configuration during in vitro maturation of mouse oocytes to clarify the relationship between oocyte maturation and mitochondrial distribution morphologically. Oocytes were recovered from 6-to 8-wk-old ICR strain female mice. Germinal vesicle (GV)-stage oocytes were divided into 4 groups and cultured: group A, oocytes collected after pregnant mare serum gonadotropin (PMSG) injection; and group B, oocytes collected after PMSG-human chorionic gonadotropin injection. Groups A and B were subdivided into 2 groups: denuded oocytes (DO) and cumulus-enclosed-oocytes (CEO). At 0, 4, 8, 12 and 16 h from the onset of the culture, oocytes were fixed and stained to visualize α-tubulin, chromatin and mt using confocal laser scanning microscopy (CLSM). It was observed that mt aggregated around the nucleus from the GV-stage through progression to germinal vesicle breakdown (GVBD). With the movement of the nucleus, mt were concentrated around the nucleus and polarized. The maturation rate (the rate of the first polar body extrusion) and the fertilization rate of CEO were significantly higher than that of DO in both groups A (p<0.01) and B (p<0.05). During the GV-stage to GVBD, the rate of mitochondrial aggregation around the nucleus tended to be high in group A(CEO). The rates of mitochondrial polarization in MI and MII oocytes were 76.1% with *in-vitro* maturation (IVM) and 86.7% with *in-vivo*-maturation, respectively; the rate was significantly higher in *in-vivo*-maturation-oocytes than in IVM-oocytes (p<0.01). From the present results it can be considered that aggregaton of mitochondria around the nucleus was essential for maturation, fertilization and development.
(J Nippon Med Sch 2003; 70: 408–415)

Key words: mouse, oocyte, maturation, mitochondria, distribution, α-tubulin, chromatin, spindle

Introduction

　Cytoplasmic and nuclear maturation of the oocyte involves a complex mechanism that includes redistribution of chromosomes and organelles, especially mitochondria (mt)[1–4]. Interest in understanding the role of mt in oocyte maturation and early embryonic development has increased. The mitochondrial transcription factor A gene is essential for mitochondrial

DNA transcription, replication, and biogenesis of mt. In mouse oocytes, mt are necessary for pronuclear migration after fertilization and further meiotic division of primary cell cycles. But recently, Raffaelli et al. reported that pronuclear transfer impaired neither the ability of reconstructed non-irradiated zygotes nor the ability of reconstructed irradiated zygotes to develop as blastocysts, which suggested that at least the mitochondrial genome was not important for development to the blastocyst stage[5]. Although the role of mt in mammalian oocytes is of interest, it has not been clarified and, to our knowledge, there are no published studies on mitochondrial kinetics in oocytes during maturation under in vitro conditions. To clarify the relationship between oocyte maturation and mitochondrial distribution morphologically, we report the change of the mitochondrial distribution of mt and α-tubulin from the germinal vesicle (GV)-stage to pronucleus fusion in mouse oocytes under in vitro conditions.

Materials and Methods

Collection of Oocytes and Gametes

Oocytes were recovered from 6-to 8-week-old ICR strain female mice. For collection of oocytes, the mice were injected with 5 IU pregnant mare serum gonadotropin (PMSG) followed by 5 IU human chorionic gonadotropin (hCG) 48 h later. GV-stage oocytes were divided into 2 groups. In group A, GV-stage oocytes were collected from ovaries 48 h after PMSG injection, while in group B GV-stage oocytes were collected from ovaries 16 h from hCG injection. Collection in both groups was achieved by cutting ovaries with needles in Hepes-buffered Waymouth MB 752/1 medium supplemented with 10 ng/ml epidermal growth factor (EGF), 0.7 mM taurine, 0.23 mM Na-pyruvate, dibutil cyclic AMP, and 5% (v/v) fetal bovine serum (m-Waymouth). Further, ovulated oocytes were obtained from oviducts 16 h after hCG injection. To harvest ovulated oocytes, oviducts were removed and cut with fine needles in HEPES-buffered human tubal fluid medium (m-HTF) with 0.4% (w/v) BSA.

In Vitro Maturation

Each group was further divided into 2 groups consisting of denuded oocytes (DO) and cumulus enclosed oocytes (CEO). Therefore, GV-stage oocytes were divided into 4 groups: group A (DO), group A (CEO), group B (DO), and group B (CEO). Each group was cultured for 16 h at 37℃ under conditions of 5% CO_2 in air using Waymouth MB 752/1 medium supplemented with 10 ng/ml EGF, 0.7 mM taurine, 0.23 mM Na-pyruvate, 75 mIU/ml follicle stimulating hormone, and 5% (v/v) fetal bovine serum. At various intervals from the onset of incubation, oocytes were observed by phase-contrast microscopy (Olympus Co., Tokyo) and morphological changes in the nucleus or the extrusion of the first polar body were used as the criterion for nuclear maturation of GV-stage oocytes.

In Vitro Fertilization and Culture

Sperm was collected from epididymides of ICR male mice in human tubal fluid medium (HTF) with 0.4% BSA and capacitated for 30 min at 37℃ in 5% CO_2 in air. Both in vivo and in vitro matured MII stage oocytes were inseminated with 4×10^5 sperm/ml in HTF containing 1% (v/v) nonessential amino acid and 0.4% BSA. At 4 h after insemination, oocytes were removed from fertilization medium to HTF containing 1% (v/v) nonessential amino acid and 0.4% BSA. From insemination, the zygotes were cultured for 4, 8, and 12 h until fixation and staining.

Fixation and Immunostaining of Oocytes and Zygotes

At 0, 4, 8, 12, and 16 h from onset of GV-stage-oocyte culture, cumulus cells around the oocytes were removed by repeated pipetting in m-Waymouth medium. Oocytes and zygotes for analysis of microtubules and chromosomes were washed three times in PBS and permeabilized by treatment for 1 h at 37℃ with medium M[6], a glycerol-based microtubule-stabilizing solution containing 25% (v/v) glycerol, 50 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM 2-mercaptoethanol, 1,000 IU/ml benzylpenicillin potassium, 50 mg/l streptomycin, pH 6.7, and 4% Triton-X-100. Oocytes were then fixed by immersion in a 2% solution of paraformaldehyde

Table 1   Maturation In Vitro of Mouse Oocytes

Group		No. of GV-Stage Oocytes	Final Stage of Oocyte Maturation	
			GVBD (%)	MII (%)
A	DO	132	11 (8.3)	85 (64.4)
	CEO	195	20 (10.2)	149 (76.4) *
	Total	327	31 (9.4)	234 (71.6)
B	DO	115	19 (16.5)	54 (47.0)
	CEO	80	7 (8.8)	52 (65.0) **
	Total	195	26 (13.3)	106 (54.4)

A: collected from ovaries 48 h after PMSG injection

B: collected from ovaries after PMSG + hCG injection (hCG injection was performed 48 h after PMSG priming)

Maturation rate = No. MII-stage oocytes/No. GV-stage oocytes

Significant differences between DO and CEO are indicated by an asterisk (P < 0.05) or two asterisks (P < 0.01)

in PBS at room temperature for 30 min. After fixation, they were maintained at 4℃ for 1~3 days. Next, the fixed oocytes were incubated for 90 min at 37℃ in 1:250 solution of a monoclonal anti-α-tubulin antibody (ICN Pharmaceuticals. Inc., USA) in PBS. After washing three times in PBS containing 0.1% BSA, oocytes were incubated for 1 h in a blocking solution containing 0.1 M glycin, 1% rabbit serum, 0.01% Triton-X-100, and 0.5% BSA. Next, they were exposed for 1 h at 37℃ to fluorescein isothiocyanate conjugated anti-IgG (Fab2) antibody Human (Rockland, Inc., USA) diluted 1:250 with PBS containing 0.5% Triton-X-100 and 0.5% BSA. Thus, microtubules having been labeled, the oocytes were washed three times in PBS with 0.1% BSA and 0.1% Triton-X-100, and twice more in PBS. Oocytes were then incubated for 30 min with propidium iodide (Sigma, USA) (50 mg/ml) to visualize chromatin. The oocytes and zygotes were washed twice in PBS containing 0.1% BSA.

Oocytes for the analysis of active mt during maturation and fertilization were not fixed but were directly incubated for 30 min with 100 nM Mito-Red® (Dojin Chemicals, Japan) solution in m-HTF.

After staining, oocytes and zygotes were mounted on slideglass and observed using CLSM (Olympus Co., Tokyo).

Statistical analysis

Data were analyzed by χ²-test. In maturation, fer-

tilizing and development rate, differences in the values were considered significant when P<0.05 and P<0.01.

Results

In Vitro Maturation of Mouse Oocytes

Collected were 522 oocytes which were supplied for in vitro maturation after being divided into two groups: oocytes recovered 48 h after PMSG injection (group A) and oocytes recovered from PMSG-treated mice followed by hCG injection 16 h later (PMSG + hCG) (group B). These groups were then further subdivided into 2 groups: denuded oocytes (DO) and cumulus enclosed oocytes (CEO), resulting in 4 groups. The recovered GV-stage oocytes were matured in waymouth medium for 16 h. **Table 1** shows the number of mature oocytes collected from ovaries after injection of PMSG or PMSG + hCG. In group A, the maturation rate (arrival rate to MII stage) of CEO was significantly higher than that of DO (p<0.01), as was the maturation rate of group B (p<0.05). In group B, also, the maturation rate of CEO was significantly higher than that of DO.

In Vitro Fertilization and Development of Mouse Oocytes

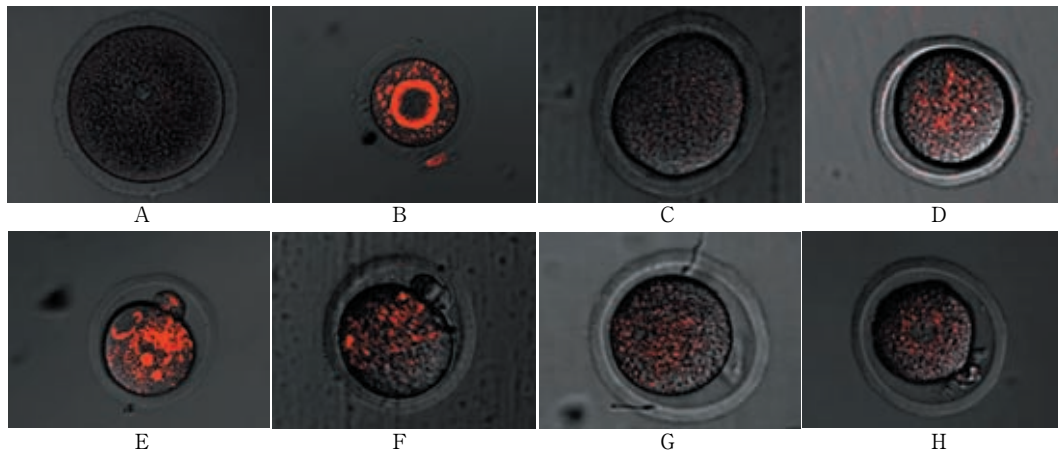Matured oocytes (MII oocytes) were subjected to in vitro fertilization. In both groups A and B, the fertili-

Fig. 1 Kinetics of Mitochondria in Mouse Oocytes

Mitochondria (red in image) were stained with Mito-Red® (100 nM) and viewed by confocal laser scanning microscopy. (A) GV-stage: mt are diffused uniformly all over the cytoplasm. (B) GV-GVBD: Aggregation of mt around the nuclei is observed. (C) MI: After GVBD, mt are dispered uniformly in cytoplasm. (D) MI: Polarization of mt is seen in cytoplasm and mt are aggregated in the part including the nucleus. (E) MII: Soon after extrusion of the first polar body, mt remain aggregated around the nucleus. (F) MII: After extrusion of first polar body, mt are still aggregated around the nucleus and make polarization. (G) Fertilized egg: After extrusion of the second polar body, mt are aggregated around the female and male pronuclei. (H) Nuclear fusion: mt are aggregated around nucleus.
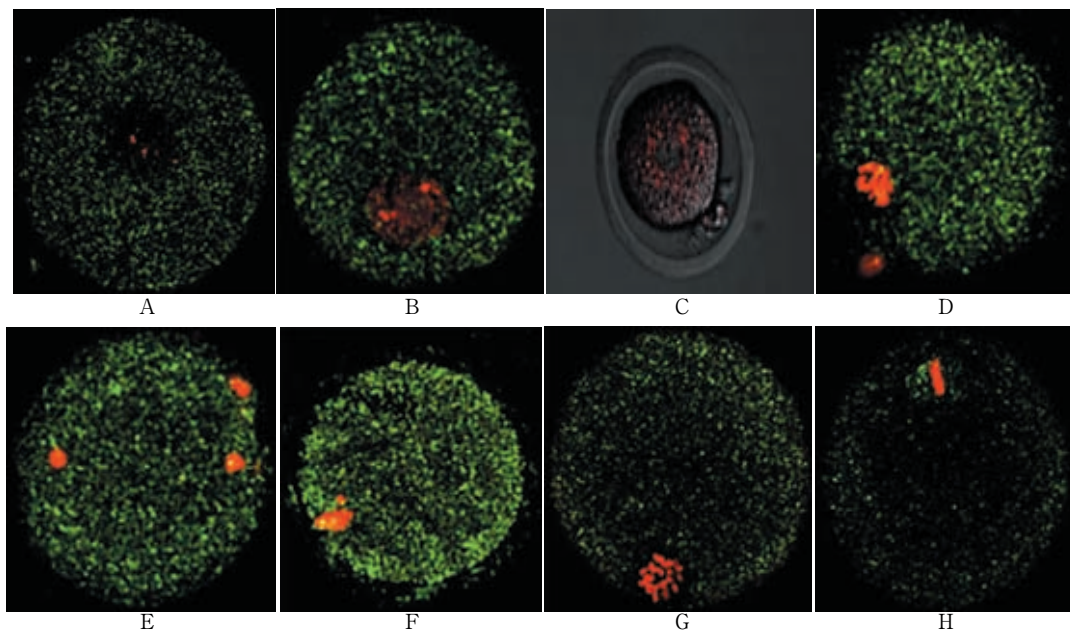

Fig. 2 Configuration of Chromatin and Spindle

α-tubulin immunostained with fluorescein isothiocyanate conjugated (FITC) anti-α-tubulin monoclonal antibody (green) and Chromosomes stained with propidium iodide (PI) (red). (A) 0 h, GV-stage: nucleus is at the center of the oocyte. (B) 8 h, MI: Chromatin has become condensed but α-tubulin is still diffused uniformly all over the cytoplasm. (C) 12 h, MI: α-tubulin is aggregated and makes spindle. Chromosomes are arranged on a plate. (D) 12 h, MII: After fertilization, the extrusion of the first polar body is seen. (E) 4 h from insemination, 2PN: After fertilization, the extrusion of the second polar body is seen and α-tubulin moves to periphery of oocyte. (F) MI arrested oocytes: Abnormal configuration with a single chromosome displaced from the plane of the metaphase plate. (G) MI arrested oocytes: Abnormal configuration of chromosomes due to absence of a spindle. (H) MI arrested oocytes: Half of the spindle is absent.

Table 2 Fertilization and Development In Vitro of Mouse Oocytes

Group			No. Matured Oocytes	No. Fertilized Oocytes (%)	No. Developed Oocytes (%)
In Vitro Maturation	A	DO	85	44 (51.7)	19 (43.2)
		CEO	149	105 (70.5) **	53 (50.5)
		Total	234	149 (63.7)	72 (48.3)
	B	DO	54	26 (48.1)	10 (38.5)
		CEO	52	33 (63.5) *	15 (45.5)
		Total	106	59 (55.7)	25 (42.3)

A: collected from ovaries 48 h after PMSG injection

B: collected from ovaries after PMSG + hCG injection (hCG injection was performed 48 h after PMSG priming)

Fertilization rate = No.fertilized oocytes/No. matured oocytes

Development rate = No. developed oocytes/No. fertilized oocytes

Significant differences between DO and CEO are indicated by an asterisk ($P < 0.05$) or two asterisks ($P < 0.01$)

Table 3 Distribution of Mitodhondria in Mouse Oocytes During Maturation In Vitro

DO		No. Oocytes	Periphery of Nuclei (%)	All over Ooplasm (%)
Stage	Group			
GV-GVBD	A	78	56 (71.8)	22 (28.3)
	B	78	48 (61.5)	30 (38.5)
M- I	A	24	17 (70.8)	7 (29.2)
	B	16	10 (62.5)	6 (37.5)
M- II	A	45	40 (88.9)	5 (11.1)
	B	24	16 (66.7)	8 (33.3)

CEO		No. Oocytes	Periphery of Nuclei (%)	All over Ooplasm (%)
Stage	Group			
GV-GVBD	A	94	76 (80.9)	18 (19.1)
	B	44	33 (75.0)	11 (25.0)
M- I	A	27	21 (77.8)	6 (22.2)
	B	16	12 (75.0)	6 (37.5)
M- II	A	57	52 (91.2)	5 (8.8)
	B	38	29 (76.3)	9 (23.7)

A: collected from ovaries 48 h after PMSG injection

B: collected from ovaries after PMSG + hCG injection (hCG injection was performed 48 h after PMSG priming)

zation rate of CEO was significantly higher than that of DO ($p < 0.01$ and $p < 0.05$, respectively) (**Table 2**). The development rate was similar between CEO and DO in both groups A and B.

Distribution of Mitochondria

The distribution of mt was visualized with Mito-Red® (**Fig. 1**). At the onset of culture, oocytes were at the GV-stage, and, in most oocytes, mt assembled around the nuclei. But in some oocytes, mt were dif- fused throughout the ooplasm. As GVBD neared, mt became more densely concentrated around the nu- cleus and this concentration was retained at GVBD. However, after GVBD, aggregates of mt disap- peared. At the MI-stage, mt again gradually aggre- gated around the nucleus and polarized through the ooplasm. During the MII-stage, mt were always con- centrated around the nucleus. After fertilization, a second polar body was extruded and aggregation of mt was seen around both the female and male pro-

nucleus. Further, at the time of nuclear fusion, aggregation of mt was observed around the nucleus.

In the analysis of distribution of mt, in 71.8% of group A (DO) oocytes, 61.5% of group B (DO) oocytes, 80.9% of group A (CEO) oocytes, and 75.0% of group B (CEO) oocytes, mt were aggregated around the nucleus from the GV-stage to GVBD (**Table 3**). In all maturation stages, the rate of mitochondrial aggregation around the nucleus tended to be high in group A (CEO).

The formation rates of mitochondrial polarization in MI and MII oocytes was 76.1% with and 86.7% with in-vivo-maturation, respectively, and was significantly higher in in-vivo-maturation-oocytes than IVM-oocytes (p<0.01).

Configuration of Chromatin and Spindle Analysis

Chromatin configuration was visualized with propidium iodide and α-tubulin was immunostained with fluorescein isothiocyanate conjugated anti-α-tubulin monoclonal antibody (**Fig. 2**). Samples were observed by CLSM. At the onset of the culture, oocytes were at the GV-stage. The nucleus was in the center of the oocyte, and α-tubulin was diffused uniformly over the cytoplasm. At 4 h from the start of culture, GVBD was observed. The nucleus became larger than that at the GV-stage. At 8 h, oocytes resumed meiotic progression. Chromatin had become condensed and well stained with propidium iodide, but α-tubulin was still diffused uniformly over the cytoplasm. At 12 h, chromatin had become condensed and separated into thick chromosomes and arranged on a plate. α-tubulin was aggregated and formed spindles. At 12 and 16 h, the first polar body was extruded and chromosomes gradually formed a mass. During *in vitro* fertilization, once fertilized, α-tubulin moved to the periphery of the oocyte. At 4 h from insemination, a second polar body was extruded and a female pronucleus and male pronucleus were observed in the oocyte. After an 8-h culture, the female nucleus and male nucleus approached each other toward the center of the oocyte, and at 12 h, fusion of pronuclei was observed. During these experiments, abnormal configuration of chromatin and spindles was observed in some IVM-oocytes.

Discussion

The role of mt in the process of cytoplasmic and nuclear maturation is of great interest but has not yet been clarified. The function of mt for early embryonic development has been reported[7-9], and the present results were in agreement with those reports that mt are necessary for pronuclear migration after fertilization and further meiotic division of primary cell cycles. In this study, we observed the change of the mitochondrial distribution in detail from the GV-stage to the pronucleus fusion stage.

In the IVM experiments, maturation and fertilization rates of CEO were significantly higher than those of DO, a result that was in accord with previous reports[10,11]. In group A (DO + CEO) maturation and fertilization, rates were significantly higher than those of group B (DO + CEO). The loss of gap junction between the cumulus cells and the zona pellucida induced by hCG injection might have resulted in the increase of DO. The poor maturation rate in group B is probably attributable to the loss of cumulus cells.

In the analysis of mitochondrial kinetics, we found that mt were always condensed around the nucleus after metaphase I. This finding is in accord with previous reports that mitochondrial maturation, distribution, ATP production and energy accumulation during oogenesis are important to cytoplasmic maturation[12-14]. Polarization was also observed in keeping with the previous report that prior to maturation, oocytes appeared to be radially symmetrical with no evident polarity and fully mature oocytes exhibited obvious polarity marked by the position of the metaphase II spindle in the cortex[15]. Oocytes in which mt were aggregated at the periphery of the nucleus from the GV-stage to GVBD were more numerous in group A than in group B. Since the maturation rate of group A was significantly higher than that of group B, the present result indicates that lack of mt aggregation at the periphery of the nucleus resulted in the arrest of oocyte maturation. The reason of mitochondrial aggregation around the nucleus is not clear, but some possibilities are suggested. The present results support the theory that perinuclear ac-

cumulation of mt provides ATP for GVBD, and the decrease in the number of mt is correlated with diminished capacity for ATP generation in human oocytes[16]. However, other research has demonstrated that meiotic maturation occurs in both mouse and human oocytes over a wide range of ATP concentrations and that the mitochondrial contribution to ATP concentration may be small in early embryonic development[17-19]. This finding is inconsistent with our results, but the discrepancy may be accounted for by clarifying other functions of mt besides ATP generation. We are currently studying this problem.

The coordination of nuclear maturation and cytoplasmic maturation is also important to oocyte maturation[20]. In previous reports on cryopreservation, damage to the spindle resulted in meiotic arrest[21,22]. In the analysis of the spindle and configuration of chromatin, deformed spindle and irregular arrangement of chromosomes were observed in some IVM-oocytes. The cause of abnormality might be due to in vitro conditions, including culture techniques. Further studies on this problem are needed.

The present study indicates the importance of structural elements including mt in the ooplasm for oocyte maturation. Elucidation of the role of mt and other cytoplasmic elements in oocytes could contribute to progress in assisted reproductive technology including IVM, in vitro fertilization, intra cytoplasmic sperm injection and cryopreservation of gametes and embryos.

References

1. Fulka JJ, First NL, Moor RM. Nuclear and cytoplasmic determinants involved in the regulation of mammalian oocyte maturation. Mol Hum Reprod 1998; 4 : 41–49.
2. Krisher RL, Bavister BD. Responses of oocytes and embryos to the culture environment. Theriogenology 1998; 49: 103–114.
3. Bavister BD. Interactions between embryos and the culture milieu. Theriogenology 2000; 53: 619–626.
4. Miodrag Stojkovic, Sergio A. Machado, Petra Stojkovic, Valeri Zakhartchenko, Peter Hutzler, Paolo B. Gonçalves, Eckhard Wolf. Mitochodrial distribution and adenosine triphosphate content of bovine oocytes before and after in vitro maturation: correlation with criteria and development capacity after in vitro fertilization and culture. Biol Repro 2001; 64: 904–909.
5. Raffaelli R, Palermo GD, Takeuchi T, Rosenwaks Z, and Bedford JM. Indirect investigation of the role of mitochondorial genes in early development. Human Reproduction 2000; 15 Abstract Books 1: 41.
6. J.L. Tremoleda, E.J. Schoevers, T.A.E. Stout, B. Colenbrander, M.M. Beavers. Organization of the cytoskeleton during in vitro maturation of horse oocytes. Mol Reprod Dev 2001; 60: 260–269.
7. Larsson N-G, Wang J, Wilhelmsson H, Oldfors A, Rustin Plewandoski M, Barsh GS, Clayton DA. Mitochondrial transcription factor A is nessesary for mt DNA maintenance and embryogenesis in mice. Nat Genet 1998; 18: 231–236.
8. Maro B, Howlett SK, Webb M. Non-spindle microtubule organizing centers in metaphase II-arrest mouse oocytes. J Cell Biol 1985; 101: 1665–1672.
9. Schatten H, Schatten G, Mazia D, Balczon R, Simerly C. Behaviour of centrosomes during fertilisation and cell division in mouse oocytes and in sea urchin eggs. Proc Natl Acad Sci 1986; 83: 105–109.
10. Eppig JJ. The relationship between cumulus cell-oocyte coupling oocyte maturation and cumulus expansion. Dev Biol 1982; 89: 268–272.
11. Stephan M. Downs, Suzan A.J. Daniel, John J. EPPING. Induction of maturation in cumulus cell-enclosed mouse oocytes by follicle-stimulating hormone and epidermal growth factor: evidence for a positive stimulus pf somatic cell origin. The Journal of zoology 1988; 245: 86–96.
12. Van Blerkom J, Runnner MN. Mitochondrial reorganization during resumption of arrested meiosis in the mouse oocyte. Am J Anat 1984; 171: 335–355.
13. Hyttel P, Greve T, Callesen H. Ultrastructure of in vitro oocyte maturation in cattle. J Reprod Fertil 1986; 38: 35–47.
14. De Loos F, Van Vliet C, Van Maurik P, Kruip TAM. Morphology of immature bovine oocytes. Gamete Res 1989; 24: 197–204.
15. Calarco PG. Polarization of mitochondria in the unfertilized mouse oocyte. Dev Genet 1995; 16 (1) : 36–43.
16. Van Blerkom J, Davis P, Alexander S. Differential mitochondrial distribution in human pronuclear embryos leads to disproportionate inheritance between blastomeres: relationship to microtubular organization, ATP content and competence Hum Reprod 2000; 15: 2621–2633.
17. Van Blerkom J, Davis P, Lee J. ATP content of human oocytes and developmental potential and outcome after in vitro fertilization and embryo transfer. Hum Reprod 1995; 10: 415–424.
18. Trimarchi JR, Liu L, Poterfiled DM, Smith PJ, Keefe DL. Oxidative phosphorylation-dependent and independent oxygen consumption by individual preimplantation mouse embryos. Biol Reprod 2000; 62: 1866–1874.
19. Van Blerkom J, Bell H. Regulation of development in the fully grown mouse oocyte: chromosome mediated temporal and spatial differentiation of the cytoplasm and plasma membrane. Journal of Embryology and Experimental Morphology 1986; 93: 213–238.

20. Catherine M.H. Combelles, David F. Albertini. Micro-tubule patterning during meiotic maturation in mouse oocytes is determined by cell cycle-specific sorting and redistribution of γ-tubulin. Dev Biol 2001; 239: 281–294.
21. Irene Boiso, Mercé Martí, Josep Santaló, Montse Ponsá, Pere N. Barri, Anna Veiga. A confocal micros-copy analysis of the spindle and chromosome con-figurations of human oocytes cryopreserved at the germinal vesicle and metaphase II stage. Hum Re-prod 2002; 17: 1885–1891.
22. Almeida P, Bolton V. The effect of temperature fluc-tuations on the cytoskeletal organization and chro-mosomal constitution of human oocytes. Zygote 1995; 3 : 357–365.