—Full Paper—

Mitochondrial Dysfunction of *In Vitro* Grown Rabbit Oocytes Results in Preimplantation Embryo Arrest After Activation

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Abstract. To clarify the mechanism that impairs development of *in vitro* grown (IVG) oocytes, we assessed whether the developmental disability of IVG oocytes is caused by cytoplasmic dysfunction. First, we assessed the cleavage of nuclear-substituted oocytes cultured *in vitro*. The nuclei, but not the cytoplasm, of the IVG oocytes were able to support subsequent cleavage after artificial activation. The mitochondrial activity of the oocytes increased as the follicles grew. However, the mitochondrial activity of the IVG oocytes was significantly lower than that of ovulated oocytes and oocytes recovered from follicles with diameters of more than 300 μm . Furthermore, the expression levels of mitochondrial transcriptional factor A (TFAM) in the oocytes increased in a similar manner. However, the expression levels of TFAM in the IVG oocytes was significantly lower than that of ovulated oocytes and oocytes recovered from follicles with diameters of more than 300 μm . Taken together, these results indicate that the low developmental competence of IVG oocytes is caused by a cytoplasm deficiency due to low mitochondrial activity.

Key words: *In vitro* growth, Growing oocytes, Mitochondria, Mitochondrial transcriptional factor A (TFAM), Rabbit

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The developmental competence of *in vitro* grown (IVG) oocytes after *in vitro* fertilization (IVF) is low compared with that of fully grown oocytes in mice [1], cattle [2] and rabbits (our unpublished data). This low developmental competence is believed to be caused by cytoplasmic dysfunction during *in vitro* growth. Oocytes contain a pool of non-replicating yet functional mitochondria from which all cells of the resultant embryos directly inherit their own complements. Ooplasmic mitochondrial metabolism has recently been shown

to be necessary for initiation [3] and progression [4] of preimplantation embryo development in the mouse. Age-related morphological changes have been reported in human oocytes [5], and these changes may correlate with the progressive age-related decline in mitochondrial membrane potential that has been reported relative to embryo cell number [6]. Furthermore, the oocyte ATP level has been shown to correlate with postimplantation outcome [7] and to be involved in both arrest and resumption of meiosis in murine oocytes [8, 9]. It has also been postulated that disordered spatial patterning of these organelles in the human oocyte could be inherited by daughter cells and result in local metabolic heterogeneities [10].

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Mitochondrial dysfunction is likely detrimental to postimplantation development because all embryonic cells require mitochondria for cytoplasmic energy production and because mitochondria are important regulators of preimplantation embryo development.

Replication of mtDNA is dependent on nuclear-encoded transcription and replication factors being translocated to the mitochondria [11]. Replication of mtDNA utilizes an RNA primer generated through transcription by mitochondrial transcription factor A (TFAM) [12] and the mitochondrial-specific polymerase gamma [13], which consists of the catalytic (POLG) and accessory (POLG2) subunits [14]. These two factors regulate the mtDNA copy number, which is correlated with the oxidative phosphorylation requirements of specific cells [15]. MtDNA replication occurs throughout oocyte growth in many species [16, 17]; however, based on observations in the frog [18], mouse [19, 20] and shrimp [21], it probably does not occur in the early cleavage stage of embryogenesis. The absence of mtDNA replication until the blastocyst stage has recently been confirmed in the mouse [22] and has also been reported in cattle [23]. However, a short burst of mtDNA turnover has been observed in mouse 2-cell embryos [24].

In the present study, the metaphase II spindles of IVG oocytes were transplanted into healthy enucleated ooplasts to determine whether the low developmental competence of IVG oocytes is caused by cytoplasmic dysfunction. The manipulated oocytes were then activated and cultured to the blastocyst stage. In addition, the mitochondrial activity of the IVG oocytes was assessed.

Materials and Methods

In vitro growth of rabbit oocytes

Multiple ovulation was induced in female New Zealand White rabbits by a single subcutaneous injection of 80-IU PMSG (Sankyo LifeTech, Kanagawa, Japan). The females were then intravenously administered 60 IU hCG (Teikoku Zoki, Tokyo, Japan) 72 h after the start of multiple-ovulation treatment [25].

Rabbit oocyte-granulosa cell complexes (OGCs) were recovered from follicles with diameters of

200–700 μm using 26-gauge needles, and oocyte-cumulus cell complexes (OCCs) were aspirated from follicles with diameters of more than 700 μm using 26-gauge needles 14 h after hCG injection. Intact oocytes with intact granulosa cells were selected and randomly assigned to each treatment. *In vitro* culture of growing oocytes was carried out as reported previously with some modifications [26]. Briefly, OGCs were recovered from follicles with diameters of less than 300 μm and cultured in minimum essential medium alpha medium (αMEM , 11900-024; Invitrogen, Carlsbad, CA, USA) supplemented with 3 mg/ml BSA (A7638; Sigma-Aldrich, St. Louis, MO, USA), 50 $\mu\text{g}/\text{ml}$ Ascorbic acid (A4544; Sigma-Aldrich), Insulin-Transferrin-Selenium-A (51300-044; Invitrogen), and 2% PVP (PVP360; Sigma-Aldrich) at 37 C under 5% CO_2 with saturated humidity for 8 days. OCCs were recovered from follicles with diameters of more than 700 μm and matured in TCM 199 (680557; Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10 ng/ml EGF (E9644; Sigma-Aldrich), and 0.1 μM estradiol-17 β (E-8875; Sigma-Aldrich) at 37 C under 5% CO_2 with saturated humidity for 12 h. The mean diameter of the growing oocytes recovered from follicles with diameters of less than 300 μm was $91.3 \pm 4.1 \mu\text{m}$. Ten percent of the cultured oocytes (100/964) survived and developed to $112.6 \pm 10.5 \mu\text{m}$ in diameters after culture. These *in vitro* grown oocytes were used in the following experiment.

Nuclear substitution of oocytes

IVG oocytes were transferred to a droplet of M2 medium containing 10 $\mu\text{g}/\text{ml}$ Hoechst 33342 (B2261; Sigma-Aldrich) and 7.5 $\mu\text{g}/\text{ml}$ cytochalasin B (C6762; Sigma-Aldrich). The chromosome-spindle complex and first polar body, which can be visualized with Hoechst 33342 under UV light, were drawn into a pipette along with a small amount of accompanying ooplasm using a Piezo drive system. Ovulated oocytes were enucleated in the same manner. The spindle of the IVG oocytes were injected into the perivitelline spaces of enucleated ovulated oocytes. The spindles of ovulated oocytes were transferred into enucleated IVG oocytes. The reconstructed oocytes were transferred and cultured in TCM 199 medium supplemented with 0.3% BSA (199BSA) containing 2 mM 6-DMAP (6-dimethylaminopurine, D2629; Sigma-Aldrich) under 5% CO_2 in air for 45 min to

reduce MPF activity. The oocytes were transferred and cultured in 199BSA under 5% CO₂ in air until electrical fusion. The reconstructed oocytes were transferred into mannitol fusion media (0.3 M mannitol, 0.01% PVA, 1.7 mM MgCl₂ · 6H₂O) at room temperature, aligned, and placed between the electrodes of an electric cell fusion processor (SSH-10; Shimadzu, Kyoto, Japan). They were then fused with two 80- μ sec pulses of 3.0 kVDC/cm, washed in 75, 50 and 25% fusion medium, washed in M2 medium, and placed in 199BSA.

Activation

The fused oocytes were placed between the wire electrodes (500 μ m apart) of an electroporator (FTC-22S; Shimadzu) in mannitol activation media (0.3 M mannitol, 0.01% BSA, 0.52 mM MgSO₄ and 0.1 mM CaCl₂), artificially activated with two 30- μ sec pulses of 2.5 kV/cm, and placed in 199BSA containing 7.5 μ g/ml cytochalasin B for 30 min. The activation procedure was repeated two or more times, and then the oocytes were placed in a drop of 199BSA containing 2 mM 6-DMAP for 2 h. Parthenogenesis was confirmed by detection of a pseudopronucleus. Parthenogenetic embryos were cultured in 199BSA.

Measurement of the mitochondrial activity of oocytes

Measurement of the mitochondrial activity of oocytes was performed as reported previously [27]. Oocytes were recovered from follicles with diameters of 200–700 μ m, washed twice, and incubated for 15 min in M2 medium containing Rhodamine 123 (50 μ g/ml; Molecular Probes, Eugene, OR, USA). They were then washed in M2 medium to remove traces of the dye, placed in a well dish with M2 medium, and observed with an inverted fluorescence microscope (High-sensitivity cooled CCD, VB-6010; Keyence, Osaka, Japan) equipped with excitation (500–524), beam splitter (520 nm), and emission (542/27–25) filter. Fluorescent JPEG images were obtained with a digital camera (Keyence), and measurements were obtained by counting the number of pixels (Fig. 1).

Measurement of the mRNA in oocytes

Follicles having diameters of 200–300, 300–500, 500–700 and more than 700 μ m, ovulated oocytes, and cultured oocytes were collected, centrifuged and stored in liquid nitrogen for RNA extraction. Total RNA was extracted using RNAqueous-Micro

(Ambion, Austin, TX, USA) according to the manufacturer's instructions. Single strand cDNA was prepared from total RNA using random hexamers under standard conditions with a Superscript III Reverse Transcriptase Kit (Invitrogen). The cDNA from each sample was used for a quantitative PCR assay for mitochondrial transcriptional factor A (TFAM). PCR reactions to obtain the sequences of the rabbit homologs were performed with total cDNA using HotMaster Taq (Eppendorf, Hamburg, Germany) and the appropriate primers. All PCR primers were designed to span a putative exon-exon junction in the cDNA to avoid amplification of genomic DNA. The reaction profile was 2 min at 94 C followed by 35 cycles of 94 C for 20 sec, 58 C for 10 sec and 70 C for 20 sec.

The primers for the sequences were designed to amplify tfam (5-ACAACTACCCATATTAAAGC-3 and 5-TATAAGCTGAACGAGGTC-3). After PCR, amplicons were cloned using the pGEM-T Easy Vector System (Promega, Madison, WI, USA) and then sequenced using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

Quantitative PCR was performed with oocyte cDNA using Perfect Real Time SYBR Green (Takara Bio, Ohtsu, Japan) and a 7300 Real-Time PCR System (Applied Biosystems). The PCR conditions were 95 C for 5 min followed by 35 cycles of 95 C for 30 sec, 60 C for 30 sec and 72 C for 30 sec. Reactions were replicated two times for three different samples prepared independently. The results were analyzed using the 7300 system SDS software (Applied Biosystems) and Microsoft Excel (Microsoft, Redmond, WA, USA). All experiments included negative controls consisting of no cDNA for each primer pair. The primers for real-time PCR were designed to span exons to distinguish cDNA from genomic DNA products. The primers were tfam (5-CCAGAGGCAAAAATTTCAGAGCTA-3 and 5-TCATATACCTTTTCTCTGAATCAGGAA-3).

Assessment of spindle structure

The shape and size of the meiotic spindle and chromosomal alignments of the IVG oocytes were imaged using β -tubulin and nuclear DNA staining as described previously [28, 29].

The oocytes were fixed individually in 4% paraformaldehyde (P6148; Sigma-Aldrich) in PBS

Table 1. Developmental competence of nuclear-substituted oocytes after activation of parthenogenesis

	No. of oocytes	No. (%) of embryos developed				
		Pronuclear stage	4-cell stage	8-16-cell stage	Morula stage	Blastocyst stage
Ovulated oocytes	23	21 (91)	18 (78)	14 (60)	13 (56)	9 (39)
Reconstituted oocytes with the cytoplasm of IVG oocytes	17	1 (5)	0 (0)	0 (0)	0 (0)	0 (0)
Reconstructed oocytes with the nuclei of IVG oocytes	20	15 (75)	10 (50)	6 (30)	4 (20)	1 (5)

Fig. 1. Mitochondrial activity of oocytes from growing follicles, ovulated oocytes (MII) and *in vitro* grown oocytes. <300: Oocytes recovered from follicles with diameters of less than 300 μm. 300–500: Oocytes recovered from follicles with diameters of 300–500 μm. 500–700: Oocytes recovered from follicles with diameters of 500–700 μm. >700: Oocytes recovered from follicles with diameters of more than 700 μm. IVG: *In vitro* grown oocytes. M2: Ovulated oocytes. ^{ab}P<0.05. **Fig. 2.** Expression levels of TFAM in oocytes from growing follicles, *in vitro*-grown (IVG) oocytes, ovulated oocytes (MI), and *in vitro* matured (IVM) oocytes. ^{abc}P<0.05.

for 2 h, treated overnight with 0.1% Triton X-100 in PBS, and incubated in blocking solution (PBS containing 5 mg/ml of BSA) for 30 min. They were then incubated with anti-β-tubulin antibody in PBS (1:200; Sigma-Aldrich) for 1 h at 37 C, washed for 1 h, and labeled with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (1:1,000; Sigma-Aldrich) in PBS-Tween for 1 h. Subsequently, they were washed, stained with 10 μg/ml DAPI for examination of chromosomes, and observed with a fluorescent microscope (Axiophot 2, Carl Zeiss, Oberkochen, Germany) with an appropriate filter set. Fluorescent images were captured, and measurements were obtained by counting the number of pixels using imaging software (Image J).

Statistical analysis was performed using Fisher's protected least significant difference (PLSD) test following analysis of variance (ANOVA) or Student's *t*-test using the StatView program, version 5.0 (SAS Institute, Cary, NC, USA).

Results

In vitro development of nuclear-substituted oocytes after activation

Reconstituted oocytes containing the cytoplasm of IVG oocytes and the nucleus of ovulated oocytes were incapable of developing after activation of parthenogenesis (Table 1). On the other hand, reconstructed oocytes containing the nucleus of IVG oocytes and cytoplasm of ovulated oocytes were capable of developing to blastocyst stage embryos after activation of parthenogenesis. Notably, reconstructed oocytes containing the cytoplasm of IVG oocytes were incapable of reaching the pronuclear stage.

Mitochondrial activity of oocytes

The mitochondrial activity of oocytes increased with increasing follicle diameter (Fig. 1). However, the mitochondrial activity of the IVG oocytes was

Fig. 3. Meiotic spindle configurations of a) ovulated oocytes, b) *in vitro*-matured oocytes, and c) reconstructed oocytes containing the nuclei of IVG oocytes and cytoplasm of ovulated oocytes. The images depict barrel-shaped spindles with normal chromosomal alignments in the oocytes, β -tubulins (green) and chromosomes (blue). Scale bars are 30 μm .

significantly lower than the mitochondrial activities of the ovulated and *in vitro*-matured oocytes ($P < 0.05$). The expression level of the mRNA of TFAM increased with increasing follicle diameter (Fig. 2). On the other hand, the expression level of the IVG oocytes was significantly lower than the levels of the ovulated oocytes and oocytes recovered from follicles with diameters of 300–500, 500–700, and more than 700 μm .

Assessment of spindle structure

The meiotic spindle configuration of the IVG oocytes after nucleus replacement was similar to that of ovulated oocytes (Fig. 3).

Discussion

The preceding results clearly indicate that developmental arrest of the IVG oocytes was caused by the low activities of their mitochondria. Furthermore, the ATP content of recipient-mouse MII-stage oocytes can be increased by mitochondrial infusion [29], and transfused mitochondria can survive embryonic development and undergo replication [30]. In addition, the nuclear replacement technique can support development of the nuclei of cells with mitochondria damage [31]. In this study, developmental arrest was reversed once the nucleus of an IVG oocyte was transplanted into ovulated ooplasm. Thus, mitochondria damaged during *in vitro* growth may cause developmental arrest in IVG oocytes. This hypothesis is supported by the finding that the mitochondrial activity of

growing oocytes increased with increasing follicle diameter; however, the mitochondrial activities of the IVG oocytes were significantly lower than those of the oocytes that were not grown *in vitro*. Replication of mtDNA is dependent on nuclear-encoded transcription and replication factors being translocated to the mitochondria [11]. Replication of mtDNA utilizes an RNA primer generated through transcription by TFAM. Quantitative analysis of TFAM also indicated that replication of mtDNA in growing oocytes increased as the follicles grew. However, the level of TFAM mRNA in the IVG oocytes was extremely low. In this study, rabbit oocytes were cultured under 20% oxygen tension. Higher oxygen tension during *in vitro* culture of mammalian embryos has been suggested to cause developmental arrest as a result of the generation of reactive oxygen species in cells [32]. Thus, the mitochondria of IVG oocytes might be damaged by *in vitro* culture under high oxygen concentration conditions.

Segregation of meiotic chromosomes/chromatids depends on their correct binding to the fibers of the meiotic spindle [33]. Although the dynamics of spindle function are unclear, the polymerization of spindle microtubules appears to depend on ATP [34, 35]. Mitochondria are the major organelles providing ATP. Mitochondrial DNA is easily damaged, and the consequence of this damage is underproduction of ATP, which results in respiratory chain defects [36]. Immunofluorescent images of the meiotic spindles and chromosomes of IVG oocytes suggested that there was sufficient ATP in the cytoplasm as a consequence of nuclear replacement. These data

suggest that the membrane potential of the mitochondria in the cytoplasm of the ovulated oocytes were intact, resulting in normal chromosomal alignments.

The results of the present study indicate that the developmental competence of the IVG oocytes was impaired by mitochondrial dysfunction due to damage to the mitochondria during *in vitro* culture.

References

1. Eppig JJ, O'Brien MJ. Development *in vitro* of mouse oocytes from primordial follicles. *Biol Reprod* 1996; 54: 197–207.
2. Hirao Y, Itoh T, Shimizu M, Iga K, Aoyagi K, Kobayashi M, Kacchi M, Hoshi H, Takenouchi N. *In vitro* growth and development of bovine oocyte-granulosa cell complexes on the flat substratum: effects of high polyvinylpyrrolidone concentration in culture medium. *Biol Reprod* 2004; 70: 83–91.
3. Dumollard R, Marangos P, Fitzharris G, Swann K, Duchen M, Carroll J. Sperm-triggered $[Ca^{2+}]$ oscillations and Ca^{2+} homeostasis in the mouse egg have an absolute requirement for mitochondrial ATP production. *Development* 2004; 131: 3057–3067.
4. Lane M, Gardner DK. Mitochondrial malate-aspartate shuttle regulates mouse embryo nutrient consumption. *J Biol Chem* 2005; 280: 18361–18367.
5. de Bruin JP, Dorland M, Spek ER, Posthuma G, van Haaften M, Looman CW, te Velde ER. Age-related changes in the ultrastructure of the resting follicle pool in human ovaries. *Biol Reprod* 2004; 70: 419–424.
6. Wilding M, Dale B, Marino M, di Matteo L, Alviggi C, Pisaturo ML, Lombardi L, De Placido G. Mitochondrial aggregation patterns and activity in human oocytes and preimplantation embryos. *Hum Reprod* 2001; 16: 909–917.
7. Van Blerkom J, Davis PW, 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.
8. Downs SM. The influence of glucose, cumulus cells, and metabolic coupling on ATP levels and meiotic control in the isolated mouse oocyte. *Dev Biol* 1995; 167: 502–512.
9. Downs SM, Mastropolo M. The participation of energy substrates in the control of meiotic maturation in murine oocytes. *Dev Biol* 1994; 162: 154–168.
10. 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.
11. Clayton DA. Transcription and replication of mitochondrial DNA. *Hum Reprod* 2000; 15 (Suppl 2) 11–17.
12. Larsson NG, Wang J, Wilhelmsson H, Oldfors A, Rustin P, Lewandoski M, Barsh GS, Clayton DA. Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. *Nat Genet* 1998; 18: 231–236.
13. Hubscher U, Kuenzle CC, Spadari S. Functional roles of DNA polymerases beta and gamma. *Proc Natl Acad Sci USA* 1979; 76: 2316–2320.
14. Gray H, Wong TW. Purification and identification of subunit structure of the human mitochondrial DNA polymerase. *J Biol Chem* 1992; 267: 5835–5841.
15. Moyes CD, Battersby BJ, Leary SC. Regulation of muscle mitochondrial design. *J Exp Biol* 1998; 201: 299–307.
16. Jansen RP, de Boer K. The bottleneck: mitochondrial imperatives in oogenesis and ovarian follicular fate. *Mol Cell Endocrinol* 1998; 145: 81–88.
17. Smith LC, Alcivar AA. Cytoplasmic inheritance and its effects on development and performance. *J Reprod Fertil Suppl* 1993; 48: 31–43.
18. El Meziane A, Callen JC, Mounolou JC. Mitochondrial gene expression during *Xenopus laevis* development: a molecular study. *EMBO J* 1989; 8: 1649–1655.
19. Ebert KM, Liem H, Hecht NB. Mitochondrial DNA in the mouse preimplantation embryo. *J Reprod Fertil* 1988; 82: 145–149.
20. Piko L, Taylor KD. Amounts of mitochondrial DNA and abundance of some mitochondrial gene transcripts in early mouse embryos. *Dev Biol* 1987; 123: 364–374.
21. Vallejo CG, Lopez M, Ochoa P, Manzanares M, Garesse R. Mitochondrial differentiation during the early development of the brine shrimp *Artemia franciscana*. *Biochem J* 1996; 314: 505–510.
22. Thundathil J, Filion F, Smith LC. Molecular control of mitochondrial function in preimplantation mouse embryos. *Mol Reprod Dev* 2005; 71: 405–413.
23. May-Panloup P, Vignon X, Chretien MF, Heyman Y, Tamassia M, Malthiery Y, Reynier P. Increase of mitochondrial DNA content and transcripts in early bovine embryogenesis associated with upregulation of mtTFA and NRF1 transcription factors. *Reprod Biol Endocrinol* 2005; 3: 65–72.
24. McConnell JM, Petrie L. Mitochondrial DNA turnover occurs during preimplantation development and can be modulated by environmental factors. *Reprod Biomed Online* 2004; 9:

- 418–424.
25. **Hashimoto S, Kimura K, Kuramochi T, Aoyagi K, Hirako M, Kawaguchi M, Iwata H, Hirao M, Kitada K, Hirasawa K, Ueda M.** Responsiveness of rabbits to superovulation treatment by single injection of follicle stimulating hormone with aluminum hydroxide gel. *Mol Reprod Dev* (in press).
 26. **Hashimoto S, Ohsumi K, Tsuji Y, Harauma N, Miyata Y, Fukuda A, Hosoi Y, Iritani A, Morimoto Y.** Growing porcine oocyte-granulosa cell complexes acquired meiotic competence during *in vitro* culture. *J Reprod Dev* 2007; 53: 379–384.
 27. **Thouas GA, Trounson AO, Wolvetang EJ, Jones GM.** Mitochondrial dysfunction in mouse oocytes results in preimplantation embryo arrest *in vitro*. *Biol Reprod* 2004; 71: 1936–1942.
 28. **Wang WH, Keefe DL.** Prediction of chromosome misalignment among *in vitro* matured human oocytes by spindle imaging with the Polscope. *Fertil Steril* 2002; 78: 1077–1081.
 29. **Van Blerkom J, Sinclair J, Davis P.** Mitochondrial transfer between oocytes: potential applications of mitochondrial donation and the issue of heteroplasmy. *Hum Reprod* 1998; 13: 2857–2868.
 30. **Barrit JA, Brenner CA, Malter HE, Cohen J.** Mitochondria in human offspring derived from ooplasmic transplantation: brief communication. *Hum Reprod* 2001; 16: 513–516.
 31. **Takeuchi T, Neri QV, Katagiri Y, Rosenwaks Z, Palermo GD.** Effect of treating induced mitochondrial damage on embryonic development and epigenesis. *Biol Reprod* 2005; 72: 584–592.
 32. **Johnson MH, Nasr-Esfahani MH.** Radical solutions and cultural problems: could free oxygen radicals be responsible for the impaired development of preimplantation mammalian embryos *in vitro*? *Bioessays* 1994; 16: 31–38.
 33. **Eichenlaub-Ritter U, Vogt E, Yin H, Gosden R.** Spindles, mitochondria and redox potential in aging oocytes. *Reprod Biomed Online* 2003; 8: 145–158.
 34. **Van Blerkom J, Runner MN.** Mitochondrial reorganization during resumption of arrested meiosis in the mouse oocyte. *Am J Anat* 1984; 171: 335–355.
 35. **Van Blerkom J.** Microtubule mediation of cytoplasmic and nuclear maturation during the early stages of resumed meiosis in cultured mouse oocytes. *Proc Natl Acad Sci USA* 1991; 88: 5031–5035.
 36. **Wallace DC.** Mitochondrial diseases in man and mouse. *Science* 1999; 283: 1482–1488.