# Chapter 2

## Maternal ageing predisposes to mitochondrial damage and loss during *in vitro* maturation of equine oocytes

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## ABSTRACT

In many mammalian species, reproductive success decreases with maternal age. This decrease has been proposed to result, at least in part, from an age-related reduction in the mitochondrial quantity and/or quality in oocytes. The aim of this study was to determine whether maternal age and *in vitro* maturation (IVM) affect the quantity and quality of mitochondria in equine oocytes. Oocytes were collected from the ovaries of slaughtered mares categorized as young (<12 years) or aged (≥12 years). The mean mitochondrial DNA copy number was estimated by quantitative PCR and found to be significantly lower in oocytes from aged mares after IVM than in any other group. No evidence of age-related deletions or mutations in the mitochondrial genome was found. Transmission electron microscopy demonstrated that mitochondria in aged mare oocytes subjected to IVM experienced significantly more swelling and damage to the cristae than those in other groups. In conclusion, maternal ageing is associated with a heightened susceptibility to mitochondrial damage and loss in equine oocytes, which manifests during maturation *in vitro*. This predisposition to mitochondrial degeneration probably contributes to the age-related reduction in reproductive success in mares and other female mammals.

## INTRODUCTION

In many mammalian species, there is a threshold maternal age beyond which reproductive success decreases markedly (Morris and Allen 2002; Heffner 2004). In women, fertility declines after 35 years of age (Menken et al. 1986), largely as a result of an associated increase in the incidence of spontaneous abortion (Nybo Andersen et al. 2000). The decline culminates in the cessation of fertility in the majority of women at around 41 years of age, even though the menopause does not onset until a mean age of 51 years (te Velde and Pearson 2002). Similarly, the likelihood of a live birth per embryo transferred in a human IVF-program decreases from 24% at maternal ages below 30 years, to 8% at 42 years and 4% at 45 years or more (Templeton et al. 1996). The underlying causes of reproductive senescence have been proposed to include a decrease in the size of the resting follicle pool, a decrease in oocyte quality, and impaired endometrial receptivity. The size of the resting follicle pool is indeed closely related to fertility in women, especially just prior to the menopause (Faddy et al. 1992; Faddy and Gosden 1995). However, a reduction in oocyte number does not explain why the majority of women are cyclic but unable to conceive in the last ten years before the menopause (te Velde and Pearson 2002). Similarly, because the negative effects of advanced maternal age on IVF success can largely be overcome by using an oocyte donated by a younger woman (≤35 years), impaired endometrial receptivity seems a relatively minor contributor to the pre-menopausal decline in female fertility (Templeton et al. 1996; Sauer 1997). Instead, it has been suggested that the primary contributor to reduced fertility is reduced oocyte quality; previous studies have reported a relatively low expression of spindle assembly checkpoint mRNA (Steuerwald et al. 2001), high incidence of spindle aberrations (Battaglia et al. 1996) and high incidence of chromosomal abnormalities (Pellestor et al. 2003; Pellestor et al. 2005) in oocytes from aged women. Similarly, intrafallopian transfer of oocytes from aged mares (20–26 years old) produced significantly fewer embryonic vesicles (31%) in inseminated young mare recipients than when the transferred oocytes were recovered from young (6-10 years old) mares (92%: Carnevale and Ginther 1995).

One of the postulated underlying causes of the age-dependent decrease in oocyte quality is a decline in mitochondrial function (Nagley and Wei 1998; Tilly 2001) similar to that observed during apoptosis. Mitochondria play several important metabolic roles in eukaryotic cells, including energy generation by oxidative phosphorylation (OXPHOS), steroid production, β-oxidation and calcium homeostasis. However, mitochondria are also implicated in processes associated with cell deterioration, such as the production of potentially harmful reactive oxygen species (ROS: Wallace 1994; Scheffler 2000). Since mtDNA is located close to the site of ROS generation, it may be particularly prone to the accumulation of oxidative damage over time. Moreover, mtDNA is more sensitive to mutagens than nuclear DNA because it lacks introns, protective histones (Wallace et al. 1987) and DNA repair mechanisms. Indeed, the mutation rate of mtDNA is more than 10 times higher than that of nuclear DNA (Wallace et al. 1987), and point mutations, deletions and duplications have been reported to accumulate in mtDNA over time, particularly in slowly or non-dividing, post-mitotic tissues with high energy demands such as brain and muscle (Cortopassi and Arnheim 1990; Shigenaga et al. 1994; Melov et al. 1995; Jazin et

al. 1996; Nagley and Wei 1998). Mammalian oocytes are also non-dividing, post-mitotic cells; however, their energy demands should be modest since they arrest after entering meiosis and remain in a resting phase until reactivation during final follicle development prior to ovulation. Nevertheless, because this resumption of meiosis may occur many years later, oocytes may, like post-mitotic somatic cells, accumulate mitochondrial DNA damage with increasing host age. However, studies that examined a possible age-related decline in oocyte mtDNA quality have produced conflicting results (Chen et al. 1995; Keefe et al. 1995; Muller-Hocker et al. 1996; Blok et al. 1997; Brenner et al. 1998; Barritt et al. 1999; Barritt et al. 2000). In particular, it has yet to be demonstrated conclusively that maternal age is related to a decrease in oocyte mtDNA quantity (Steuerwald et al. 2000; Reynier et al. 2001; Barritt et al. 2002). Critically, several of the earlier studies were hampered by the use of relatively small and biased populations of oocytes obtained from aged women attending IVF clinics because of fertility problems. Furthermore, the oocytes available for analysis were predominantly those considered unsuitable for transfer because of fertilisation failure.

The aim of the present study was to determine whether maternal age significantly affects the quality of equine oocytes before and/or after maturation *in vitro*, in terms of mtDNA copy number, mtDNA sequence deletions or mutations, and the normality of mitochondrial morphology. The mare is an attractive animal model, because the horse is a monovulatory species in which fertility decreases markedly with advancing maternal age (Morris and Allen 2002), the time interval to reproductive senescence is more comparable to women than in laboratory species (e.g. mouse), and oocytes can be obtained from slaughtered animals.

## MATERIALS AND METHODS

### Collection and culture of cumulus oocyte complexes

Immediately after slaughter, the ovaries were recovered from 268 mares. Age was estimated on the basis of standard parameters for dental eruption and wear described for horses (Muylle et al. 1996; Lowder and Mueller 1998). Animals for which the age could not be estimated reliably, due for example to missing dental elements, dental malformation, abnormal attrition or dental disease, were excluded from the study. After recovery, the ovaries of young mares (<12 years old) and aged mares (≥12 years) were transported separately to the laboratory in thermos flasks at approximately 30°C. Upon arrival at the laboratory within 4 hours of slaughter, the ovaries were washed with tap water (30°C) and any extraneous tissue was removed. The ovaries were subsequently maintained at 30°C in a 0.9% (w/v) saline solution supplemented with 0.1% (v/v) penicillin/streptomycin (Gibco BRL, Life Technologies, Paisley, UK). Cumulus oocyte complexes (COCs) were then recovered and, either immediately prepared for storage, or cultured as previously described by Tremoleda *et al.* (2001), with minor modifications. COCs were collected by aspirating the contents of 5-30 mm follicles using a 16 gauge short-bevel needle attached via an infusion set to a 250 ml polypropylene collection tube (Corning Incorporated–Life

Sciences, Big Flats, New York, USA) which was, in turn, connected to an adjustable vacuum pump. To increase the likelihood of COC recovery, after aspiration the follicle lumen was flushed 2-3 times in rapid succession with 0.9% (w/v) saline solution supplemented with 0.1% (w/v) bovine serum albumin (BSA; Sigma-Aldrich Chemicals BV, Zwijndrecht, the Netherlands) and 25 IU/ml heparin (Leo Pharmaceutical, Weesp, The Netherlands) while the follicle wall was scraped vigorously with the bevel of the needle. The recovered fluid was then allowed to stand for approximately 10 minutes at room temperature so that the COCs could settle to the bottom of the tube. The sediment containing the COCs was then recovered and washed twice with HEPES-buffered synthetic Human Tubal Fluid (Q-HTF; BioWhittaker, Verviers, Belgium) supplemented with 0.4% BSA, after which the COCs were isolated by searching the sediment with a stereomicroscope. For each mare age group, recovered oocytes were divided randomly into 2 groups. In the first group (young, n = 127; aged, n = 106), oocytes were denuded of their cumulus cells (not-IVM) by vortexing them for 4 min in calcium and magnesium free Earles's Balanced Salt Solution (EBSS) containing 0.25% (v/v) trypsin-EDTA (Gibco BRL, Life Technologies, Paisley, UK). After vortexing, oocytes were examined under an inverted stereomicroscope to confirm that there were no remaining cumulus cells; incompletely denuded oocytes were discarded. In the second group (young, n = 114; aged, n= 154), COCs were matured *in vitro* (IVM) before denudation and storage. Prior to IVM, these COCs were washed twice in 38ºC prewarmed HEPES-M199 and once in a maturation medium consisting of M199 tissue culture medium supplemented with 10% FCS, 0.01 units/ml porcine FSH and 0.01 units/ml equine LH (Sigma-Aldrich Chemicals BV). Oocytes were then matured by incubating them in groups of 10-25 in 500 µl of maturation medium in 4-well plates (Nunc A/S, Roskilde, Denmark) for 30 h at 38.7°C in a humidified atmosphere of 5% $CO_2$-in-air. After denudation, IVM oocytes were examined with an inverted stereomicroscope to confirm that cumulus removal was complete and to determine whether a first polar body was visible. The IVM oocytes were then divided into those that had clearly reached the M-II stage, i.e. they had a visible first polar body (M-II), and those that had not (not-M-II). In all cases, after denudation and examination, oocytes were washed three times in PBS containing 0.1% (w/v) PVA (polyvinyl alcohol; Sigma-Aldrich Chemicals BV) and three times in TE, consisting of 10 mM Tris (MP Biomedicals Inc., Eschwege, Germany) and 0.1 mM EDTA (BDH Ltd., Poole, UK) in double distilled water, before they were placed individually in eppendorf tubes in 5 µl of TE and stored at –20°C until further analysis.

**DNA extraction**

Oocytes were lysed by adding 5 µl of an alkaline lysis buffer (200 mM KOH and 50 mM dithiothreitol), incubating them at 65˚C for 10 minutes and then vortexing. After addition of 5 µl of neutralisation buffer (0.9 M Tris-HCl, 0.3 M KCl and 0.2 M HCl), the lysates were further diluted to a total volume of 150 µl and stored at -20°C.

**Preparation of reference samples and internal controls**

Two series of reference samples were produced to ensure that values obtained in different quantitative PCR (QPCR) plates were comparable. First, a DNA sequence of 428 bp spanning the fragment used for QPCR was amplified (Table 1: #1) and purified using the Qiaquick Purification Kit (Qiagen, Venlo, the Netherlands). The copy number of the PCR was determined by measuring absorbance at 260 nm. Although after quantitative PCR, serial dilutions gave a linear standard curve, results for the lower concentrations became less reliable after storage at -20ºC. Therefore, a stock of lysed oocytes enriched with purified genomic DNA isolated from peripheral equine lymphocytes was prepared. Tenfold serial dilutions were amplified by QPCR and calibrated using freshly prepared dilutions of the PCR product described above and analyzed on the same microtiter plate. This reference series was reproducible after a period of storage at -20°C and was aliquoted, stored at -20°C and subsequently used on all microtitre plates. Internal assay controls were prepared by pooling a number of lysed denuded equine oocytes and storing aliquots at -20ºC either undiluted (IC-high) or after 10-fold dilution (IC-low).

**Determining oocyte mtDNA copy number by quantitative PCR**

Quantitative PCR (QPCR) was performed in 96-well plates using a real-time PCR detection system (MyiQ Single-color Real-Time PCR Detection System; Bio-Rad Laboratories, Veenendaal, the Netherlands). To minimize plate dependent effects, 96-well plates included triplicate samples of the 2 negative controls (water), the 2 internal controls (IC-high and IC-low), the 6 reference samples, and 11 not-IVM oocytes and 11 IVM oocytes. The location of the not-IVM (n = 233) and IVM samples (n = 268) was alternated in successive plates.
PCR primers (Table 1: #2) were designed for the quantification of oocyte mtDNA content. The PCR reaction mixture contained 1 µl of 1:150 diluted oocyte lysate, 2.5 µl GeneAmp® 10x PCR Gold Buffer (Applied Biosystems, Nieuwerkerk a/s IJssel, the Netherlands), 2mM $MgCl_2$ (Applied Biosystems), 10pM fluorescein (Bio-Rad), 5 µl of a 1:20,000 dilution of SyberGreen (Bio Wittaker, Inc., Walkersville, MD, USA), 0.2mM of each dNTP (Promega Benelux BV, Leiden, the Netherlands), 0.4mM of each primer (Isogen Bioscience BV, Maarsen, the Netherlands) and 0.625 IU Amplitaq Gold (Applied Biosystems) made up to a total volume of 25 µl with double distilled water. After denaturation by heating to 95˚C for 5 min, 40 PCR cycles consisting of incubation at 95˚C for 20 s, 67.7˚C for 30 s and 72˚C for 30 s, were followed by a further 5 min at 72˚C. The PCR product was checked by melting curve analysis, gel electrophoresis and sequencing of the amplicons. To determine mtDNA copy number in each oocyte, sample threshold cycles were plotted against DNA concentration. The internal control samples (IH-low and IC-high) were used to monitor the intra- and inter-assay coefficients of variation which were 13.1% and 26.7%, respectively; i.e., similar to those reported previously (Reynier et al. 2001; Bhat and Epelboym 2004).

**TABLE 1.** Primer pairs used for mtDNA quantification, detection of deletions and mutations, and the production of a reference PCR product.

| ID | Forward primer (5'-3') | Position* | Reverse Primer (5'-3') | Position* | $T_a$ | Purpose |
|---|---|---|---|---|---|---|
| #1 | AAGAAAACCCCACAAAACTA | 14051 | GTGAATGAAGAGGCAGATAAAA | 14478 | 55°C | Reference PCR product |
| #2 | CATGATGAAACTTCGGCTCCCT | 14273 | TGAGTGACGGATGAGAAGGCAG | 14390 | 67.7˚C | mtDNA quantification |
| #3 | GTTCAGACCGGAGTAATCCAGG | 2528 | AGGATTGGTGCGATGATGAATA | 3000 | 62˚C | Detection of deletions and mutations |
| #4 | TCCAATCCTTTATCAACACC | 6042 | AGGTTTGGTTGAGTGTGTA | 6586 | 58°C | Detection of deletions |
| #5 | CACCATCAACACCCAAAGCT | 15424 | CCTGAAGAAAGAACCAGATGC | 15862 | 58°C | Detection of mutations |

*bp position in equine mtDNA (GenBank entry: NC_001640); $T_a$: Annealing temperature.

**Other PCR reactions and sequencing**

Two primer pairs (Table 1: #3 and #4) enclosed a DNA region with two direct sequence repeats (12 and 13bp, respectively), since such repeats are known to predispose to mtDNA deletions (Johns et al. 1989; Schon et al. 1989; Shoffner et al. 1989; Van Tuyle et al. 1996). A third primer pair (Table 1: #5) amplified a sequence within the D-loop, the most variable region of the mtDNA; this amplicon did not include any >10bp repeats. PCR amplification was performed in standard conditions using the following protocol: denaturation at 94˚C for 2 min, 35 cycles of 94˚C for 30 s, annealing at the appropriate temperature (Table 1) for 45 s and 72˚C for 30 seconds, followed by 72˚C for 2 min. The PCR product sizes were analyzed by agarose gel electrophoresis and sequencing from both ends using the Cy5 Big Dye terminator kit (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands) and the ABI Prism 3100 sequence apparatus, as described by the manufacturer. Only sequences without background noise were examined for the presence of mutations and/or mutation heteroplasmy.

**Electron microscopic assessment of mitochondrial morphology**

Oocyte mitochondrial morphology was examined by transmission electron microscopy (TEM). Directly after collection or after *in vitro* maturation, COCs were fixed for 35-65 h in Karnovsky fixative (2% paraformaldehyde, 2.5% glutaraldehyde, 0.08 M Na-cacodylate buffer (pH 7.4), 0.25 mM $CaCl_2$ and 0.5 mM $MgCl_2$) without prior denudation. After fixation, the oocytes were washed for 10 min in 0.1 M Na-cacodylate buffer (pH 7.4) and immersed for 2 periods of 1 h in 2% osmium tetroxide in the same buffer. Subsequently, the samples were stained for 1 h with 2% aqueous uranyl, before being dehydrated by passage through a graded series of acetone solutions, and then embedded in Durcupan ACM (Fluka, Buchs, Switzerland). At intervals of 50 µm throughout each block, exploratory semithin sections (1 µm) were cut using a glass knife on a Reichert Ultracut S microtome (Leica Microsystems B.V., Rijswijk, the Netherlands). These sections were stained with toluidine blue and screened for the presence of the COC by light microscopy. If the COC was detected, further semithin sections were cut at 10 µm intervals until the maximum diameter of the COC was reached. Subsequently, ultrathin sections (50 nm) were cut with a diamond knife and mounted on single-slot formvar-carbon-coated copper grids. After staining with lead citrate for 2 min, the sections were examined and electron micrographs were taken at random locations within the equatorial plane of the oocyte via an electron microscope (Philips CM 10; Philips, Eindhoven, the Netherlands) using 80 kV and magnifications of approximately 3,000x for general overviews and 30,000x for more detailed morphological analysis. Negatives were scanned with a Linoscan 1450 scanner (Heidelberger Druckmaschinen AG, Heidelberg, Germany) at a resolution of 600 dpi. The micrographs were subsequently used to examine and describe mitochondrial morphology, while mitochondrial size was measured by hit point analysis (Griffiths 1993).

### Statistical analysis

Statistical analysis was performed using SPSS 12.0.1 for Windows (SPSS Inc., Chicago, IL, USA). The influence of maternal age, oocyte maturation stage and the interaction between these two variables on the (natural logarithm of the) mtDNA copy number was examined by ANOVA. A post-hoc Bonferroni test was used to determine which of the (maternal age x maturation stage) groups had significantly different mtDNA copy numbers. Differences in mitochondrial diameter between groups were also analysed using ANOVA and a post-hoc Bonferroni test. Normality of distribution for the mitochondrial diameter and for the natural logarithms of mtDNA copy number was confirmed in a quantile-quantile (qq) plot; in a normally distributed data set, this plot tends to a straight line. Differences between groups were considered statistically significant if $P < 0.05$.

## RESULTS

### Quantity of oocyte mtDNA

In total, 501 oocytes were analyzed for mtDNA quantity (Fig 1). The mean mtDNA copy number per oocyte was $1.13 \times 10^6$, but variation between individual oocytes was considerable (sem = $3.53 \times 10^4$: range = $4.68 \times 10^3$ to $3.82 \times 10^6$).

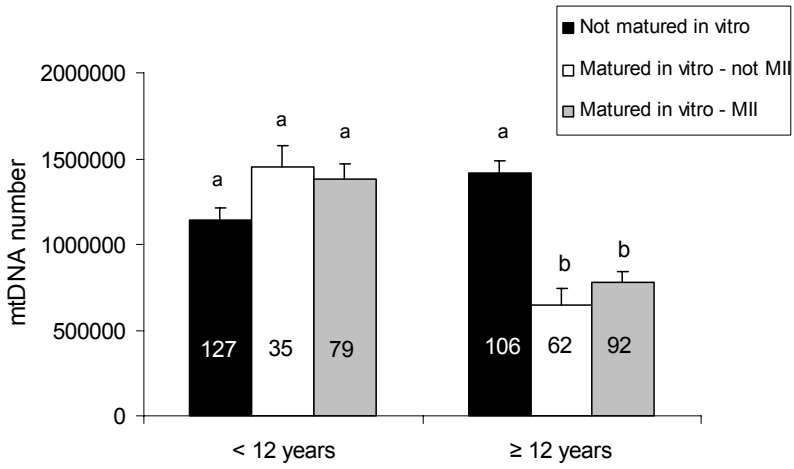


**FIG. 1.** Mean (± sem) mtDNA copy number in oocytes recovered from young (<12 years) and aged (≥12 years) mares, as determined by quantitative PCR. Within age classes**,** the oocytes are divided into three groups: (i) those that were not matured *in vitro*, (ii) those that were matured *in vitro* but did not reach M-II and (iii) those that were matured *in vitro* and did reach the M-II stage. The number of oocytes per group is indicated on the column. [a,b]Groups with different superscripts had significantly different mtDNA copy numbers ($P < 0.001$).

No significant differences in mtDNA copy number were found between oocytes recovered from young mares irrespective of whether they had been processed immediately after recovery (not-IVM: 1.15 x $10^6$) or had been matured *in vitro* and had either reached the M-II stage (IVM/M-II: 1.38 x $10^6$) or not (IVM/not-M-II: 1.46 x $10^6$). Oocytes recovered from aged mares and not subjected to maturation *in vitro* contained similar numbers of mtDNA copies (1.41 x $10^6$) as oocytes from young mares. By contrast, mtDNA copy numbers in both groups of oocytes recovered from aged mares and incubated *in vitro* (IVM/not-M-II: 0.65 x $10^6$; IVM/M-II: 0.78 x $10^6$) were significantly lower than in all other groups ($P<0.001$).

**Deletions in oocyte mtDNA**

In total, 347 oocytes were analysed for the presence of deletions; these included 162 oocytes recovered from young mares (79 not-IVM and 83 IVM oocytes) and 185 from aged mares (103 not-IVM and 82 IVM oocytes). Since most deletions described for mtDNA have been flanked by direct repeat sequences (Johns et al. 1989; Schon et al. 1989; Shoffner et al. 1989; Van Tuyle et al. 1996), we attempted to detect deletions in horse mtDNA by amplifying DNA segments containing two copies of a direct repeat in close proximity: a 12 bp sequence found at mtDNA positions 2622-2633 (GenBank entry NC_001640) and 2924-2935, and a 13-bp sequence located at positions 6207-6219 and 6384-6386. In addition, a segment of the control region was amplified. Despite amplification of DNA extracted from 347 oocytes, no deletions were detected in any of these regions. Furthermore, sequencing the mtDNA regions 2550-2978 and 15444-15841 in the mtDNA of 48 oocytes (12 not-IVM oocytes from young mares, 12 not-IVM oocytes from aged mares, 12 IVM oocytes from young mares and 12 IVM oocytes from aged mares) did not indicate any heteroplasmy as a result of somatic mutations (Jazin et al. 1996).

**Oocyte mitochondrial morphology**

Oocyte mitochondrial morphology was examined in 20 oocytes (5 per group for young versus aged combined with not-IVM versus IVM). Mitochondria in oocytes from young mares and in aged mare oocytes that had not been matured *in vitro* were comparable in size and morphology (Fig. 2A-C) with intact mitochondrial membranes, clearly appreciable transverse cristae and a mitochondrial matrix of moderate electron density. By contrast, mitochondria in IVM oocytes from aged mares had a swollen appearance (Fig. 2D). This swelling was confirmed by hit point analysis, which revealed a significantly larger mean surface area than found for mitochondria in not-IVM oocytes from both young and aged mares (Table 2). In addition, the internal architecture of the mitochondria in three out of five aged mare IVM oocytes was severely disrupted with hardly any transverse cristae visible and a relatively low mitochondrial matrix density (Fig. 2D).

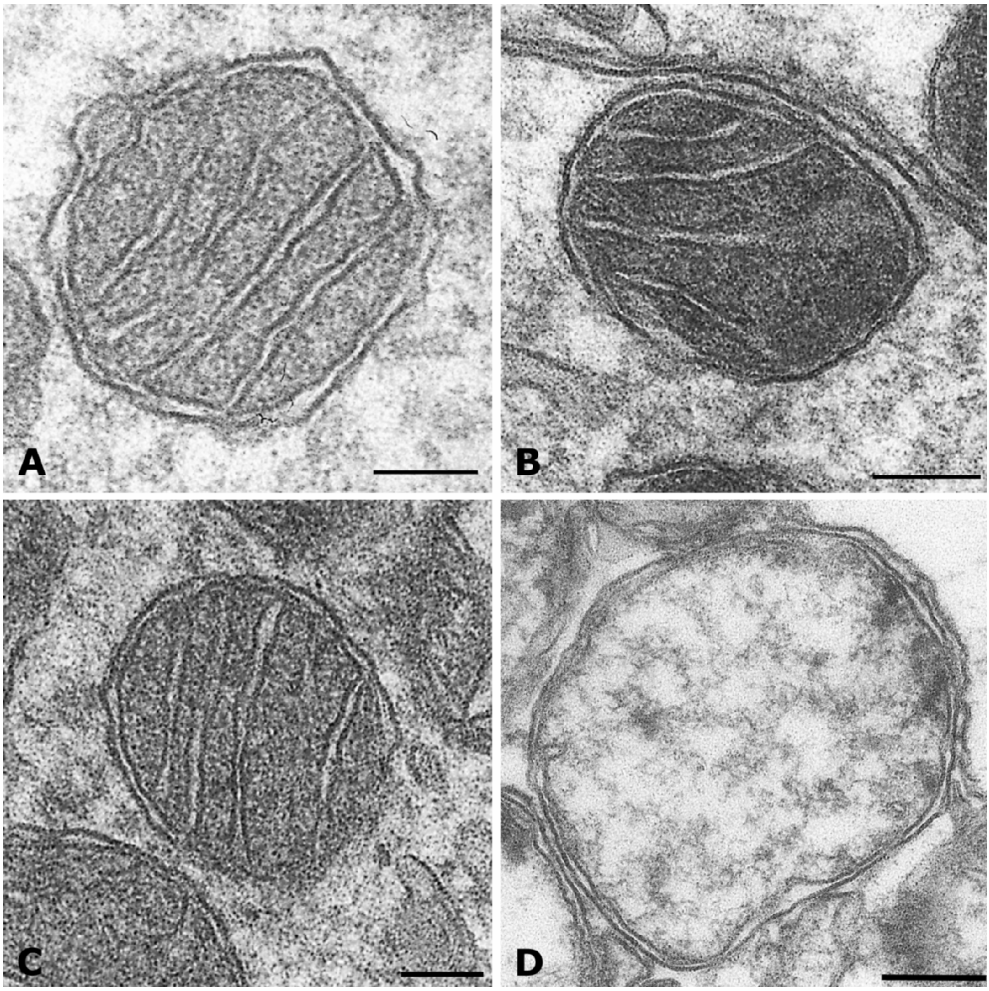


**FIG. 2.** Transmission electron micrographs of mitochondria in equine oocytes. **A** and **B)** Mitochondria in young mare oocytes that were not (A) or were (B) matured *in vitro*. The mitochondria have intact membranes and transverse cristae. **C)** A mitochondrion in an aged mare's oocyte that had not been matured *in vitro*. Its appearance is comparable to that of mitochondria in the oocytes of young mares. **D)** A mitochondrion in an aged mare's oocyte that had been matured *in vitro*. The mitochondrion is grossly swollen, has lost its transverse cristae and has a low mitochondrial matrix density. (Scale bar = 200nm).

**TABLE 2.** Relative size of mitochondria in equine oocytes, as determined by hit point analysis.

| Maternal age | Oocyte maturation stage | Mitochondrial size* |
|---|---|---|
| <12 years | Not-IVM | 40.6[a] |
| <12 years | IVM | 61.3[a,b] |
| ≥ 12 years | Not-IVM | 53.1[a] |
| ≥ 12 years | IVM | 99.7[b] |

*Relative mitochondrial size in hit points per mitochondrion (n = 5 COCs/group).
[a,b]Values with different superscripts differ significantly ($P < 0.05$).
IVM = *in vitro* matured.

## DISCUSSION

In this study, we used quantitative PCR and TEM to demonstrate that maternal age predisposes to mitochondrial degeneration in equine oocytes. In fact, oocyte mitochondrial degeneration only manifested after *in vitro* maturation of the oocytes from aged mares, and it was characterized by damage, swelling and loss of the mitochondria. In essence, these results echo the previous finding that the mitochondria in matured oocytes from aged mice are more sensitive to experimentally induced damage than those in M-II oocytes from prepubertal animals (Thouas et al. 2005). The cause of the increased susceptibility to oocyte mitochondrial damage with increasing maternal age may include a role for ROS produced by the mitochondria during OXPHOS. ROS are able to damage various cellular components including proteins, lipid membranes and DNA, and the damage inflicted on cellular structures can accumulate during an individual's lifetime (Lee and Wei 2001). Indeed, mitochondria in somatic cells of aged rats have been reported to exhibit increased oxidative stress and to allow more rapid accumulation of oxidant-induced damage to proteins and lipids than mitochondria from younger individuals (Judge et al. 2005). On the basis of the current findings and previous studies in aged women (de Bruin et al. 2004) and mice (Thouas et al. 2005), it appears that mitochondria in oocytes in the resting ('non-recruited') pool may accumulate ROS-induced damage during a female's lifetime as a result of constitutive OXPHOS. This damage may be subtle or 'subclinical' and only become apparent when the metabolic demands of the oocyte increase after the onset of follicle growth and oocyte maturation (Zeilmaker and Verhamme 1974; Magnusson et al. 1977; Magnusson and LeMaire 1981; Sutton et al. 2003). The damaged mitochondria with an impaired OXPHOS pathway may not be able to meet the increased demands for energy, leading the oocyte to enter a vicious circle of spiraling ROS production and mitochondrial damage that culminates in acute severe oxidative stress characterized by swelling, rupture and loss of mitochondria (Takeyama et al. 1993; Bernardi et al. 1999; Sastre et al. 2000).
We did not find any evidence of mtDNA damage in DNA regions predisposed to deletions by the presence of direct repeats (Johns et al. 1989; Schon et al. 1989; Shoffner et al. 1989; Van Tuyle et al. 1996). By contrast, accumulation of several aberrations, including the large so-called 'common deletion' and several point mutations, have been described to

accumulate in human mtDNA in various cell types (Cortopassi and Arnheim 1990; Shigenaga et al. 1994; Melov et al. 1995; Jazin et al. 1996; Nagley and Wei 1998) including oocytes (Chen et al. 1995; Keefe et al. 1995; Muller-Hocker et al. 1996; Blok et al. 1997; Brenner et al. 1998; Barritt et al. 1999; Barritt et al. 2000).

In the current study, mtDNA copy number did not differ between M-II and not-M-II oocytes in either young or aged mares. A similar finding was reported for human oocytes (Barritt et al. 2002), and it suggests strongly that mitochondrial replication in the female germ cell line (Jansen and de Boer 1998) is completed before the oocyte embarks on its final maturation. Furthermore, since ATP content does not differ between *in vitro* matured mouse oocytes at the GVB (germinal vesicle break-down), M-I and M-II stages (Van Blerkom et al. 1995), it appears that neither an increase in mitochondrial number nor activity is required for final oocyte maturation.

The significance of the predisposition to mitochondrial damage and loss in the oocytes of aged mares relates to the likely consequences for oocyte developmental competence, i.e. the ability to yield a viable embryo/pregnancy. For example, low mtDNA copy numbers have been associated with reduced oocyte fertilisability in women (Reynier et al. 2001) and sows (El Shourbagy et al. 2006), while extensive mitochondrial damage has been shown to impair normal germinal vesicle break down, meiotic spindle formation and chromosome segregation (Takeuchi et al. 2005). In addition, oocyte mitochondrial dysfunction has been linked to aberrant meiotic spindle function, abnormal chromosome disjunction and an increased incidence of maternally-inherited chromosomal abnormalities (Eichenlaub-Ritter et al. 2004). Low energy production (Van Blerkom et al. 1995) and mitochondrial damage (Thouas et al. 2004; Thouas et al. 2005;) in pre-ovulatory oocytes have also been associated with poor embryo development and increased pre-blastulation embryonic death. Moreover, the incidence of meiotic spindle aberrations (Eichenlaub-Ritter 1989; Battaglia et al. 1996; Liu and Keefe 2002) and chromosomal abnormalities in both oocytes (Pellestor et al. 2003; Pellestor et al. 2005;) and blastocysts (Munne et al. 1995) are higher in aged than younger females.

Critically, an embryo's mitochondria are almost exclusively maternally inherited (i.e. oocyte derived: Hutchison et al. 1974; Giles et al. 1980; Kaneda et al. 1995; Sutovsky et al. 1999) and, post-fertilisation, it is thought that embryonic mitochondrial replication is not initiated until the early gastrula stage (Piko and Taylor 1987; Ebert et al. 1988; Larsson et al. 1998; Thundathil et al. 2005). Larsson *et al.* (1998) illustrated this concept by disrupting Tfam, a gene critical for mitochondrial replication, in mice; although embryos homozygous for the disrupted gene reached gastrulation, they died soon afterwards. Given the late onset of mitochondrial replication, early embryonic cell divisions must involve the partitioning of a finite number of oocyte-derived mitochondria over an exponentially increasing number of blastomeres. Furthermore, all of the energy requirements during early embryonic development up to gastrulation must be met by this increasingly sub-divided pool of mitochondria. The decrease in mitochondrial quantity detected in the current study in aged mares oocytes subjected to IVM could easily result in insufficient mitochondria per embryonic cell to support development up to gastrulation, and thereby predispose to early embryonic loss. Indeed, aged mares are known to suffer a higher incidence of early pregnancy loss than younger individuals (Morris and Allen 2002). Reduction of the number

of mitochondria per cell before gastrulation may also explain aberrant chromosomal segregation during mitosis (Eichenlaub-Ritter et al. 2004) and karyotypic abnormalities (Hassold and Hunt 2001). Such aneuploid and mixoploid embryo mosaics have been described for many mammalian species, including man (Munne et al. 1994; Bielanska et al. 2002), rabbit (Fechheimer and Beatty 1974), pig (McFeely 1967; Long and Williams 1982), sheep (Murray et al. 1986), cow (Hare et al. 1980; Viuff et al. 1999) and horse (Rambags et al. 2005).

In conclusion, the present study demonstrates conclusively that maternal ageing predisposes to mitochondrial damage and loss, at least during equine oocyte maturation *in vitro*. It appears that when an aged oocyte reactivates, damage to the mitochondrial membranes triggers the cascade leading to swelling and loss of the mitochondrion; however, the process appears to be independent of pre-existing mtDNA sequence damage. This predisposition to mitochondrial damage may contribute to the age-dependent decrease in fertility in female mammals.

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## REFERENCES

Barritt JA, Brenner CA, Cohen J, Matt DW. 1999. Mitochondrial DNA rearrangements in human oocytes and embryos. Mol Hum Reprod 5(10):927-933.

Barritt JA, Cohen J, Brenner CA. 2000. Mitochondrial DNA point mutation in human oocytes is associated with maternal age. Reprod Biomed Online 1(3):96-100.

Barritt JA, Kokot M, Cohen J, Steuerwald N, Brenner CA. 2002. Quantification of human ooplasmic mitochondria. Reprod Biomed Online 4(3):243-247.

Battaglia DE, Goodwin P, Klein NA, Soules MR. 1996. Influence of maternal age on meiotic spindle assembly in oocytes from naturally cycling women. Hum Reprod 11(10):2217-2222.

Bernardi P, Scorrano L, Colonna R, Petronilli V, Di Lisa F. 1999. Mitochondria and cell death. Mechanistic aspects and methodological issues. Eur J Biochem 264(3):687-701.

Bhat HK, Epelboym I. 2004. Quantitative analysis of total mitochondrial DNA: competitive polymerase chain reaction versus real-time polymerase chain reaction. J Biochem Mol Toxicol 18(4):180-186.

Bielanska M, Tan SL, Ao A. 2002. High rate of mixoploidy among human blastocysts cultured in vitro. Fertil Steril 78(6):1248-1253.

Blok RB, Gook DA, Thorburn DR, Dahl HH. 1997. Skewed segregation of the mtDNA nt 8993 (T-->G) mutation in human oocytes. Am J Hum Genet 60(6):1495-1501.

Brenner CA, Wolny YM, Barritt JA, Matt DW, Munne S, Cohen J. 1998. Mitochondrial DNA deletion in human oocytes and embryos. Mol Hum Reprod 4(9):887-892.

Carnevale EM, Ginther OJ. 1995. Defective oocytes as a cause of subfertility in old mares. Biol Reprod Mono 1:209-214.

Chen X, Prosser R, Simonetti S, Sadlock J, Jagiello G, Schon EA. 1995. Rearranged mitochondrial genomes are present in human oocytes. Am J Hum Genet 57(2):239-247.
Cortopassi GA, Arnheim N. 1990. Detection of a specific mitochondrial DNA deletion in tissues of older humans. Nucleic Acids Res 18(23):6927-6933.
de Bruin JP, Dorland M, Spek ER, Posthuma G, van Haaften M, Looman CW, te Velde ER. 2004. Age-related changes in the ultrastructure of the resting follicle pool in human ovaries. Biol Reprod 70(2):419-424.
Ebert KM, Liem H, Hecht NB. 1988. Mitochondrial DNA in the mouse preimplantation embryo. J Reprod Fertil 82(1):145-149.
Eichenlaub-Ritter U. 1989. The spindle of mammalian oocytes: alterations in morphology and progression through meiosis with maternal age. Prog Clin Biol Res 311:199-220.
Eichenlaub-Ritter U, Vogt E, Yin H, Gosden R. 2004. Spindles, mitochondria and redox potential in ageing oocytes. Reprod Biomed Online 8(1):45-58.
El Shourbagy SH, Spikings EC, Freitas M, St John JC. 2006. Mitochondria directly influence fertilisation outcome in the pig. Reproduction 131(2):233-245.
Faddy MJ, Gosden RG. 1995. A mathematical model of follicle dynamics in the human ovary. Hum Reprod 10(4):770-775.
Faddy MJ, Gosden RG, Gougeon A, Richardson SJ, Nelson JF. 1992. Accelerated disappearance of ovarian follicles in mid-life: implications for forecasting menopause. Hum Reprod 7(10):1342-1346.
Fechheimer NS, Beatty RA. 1974. Chromosomal abnormalities and sex ratio in rabbit blastocysts. J Reprod Fertil 37(2):331-341.
Giles RE, Blanc H, Cann HM, Wallace DC. 1980. Maternal inheritance of human mitochondrial DNA. Proc Natl Acad Sci U S A 77(11):6715-6719.
Griffiths G. 1993. Chapter 11: Quantitative Aspects of Immunocytochemistry. In: Griffiths G, editor. Fine Structure Immunocytochemistry. Berlin, Germany: Springer-Verlag. pp 371-441.
Hare WC, Singh EL, Betteridge KJ, Eaglesome MD, Randall GC, Mitchell D, Bilton RJ, Trounson AO. 1980. Chromosomal analysis of 159 bovine embryos collected 12 to 18 days after estrus. Can J Genet Cytol 22(4):615-626.
Hassold T, Hunt P. 2001. To err (meiotically) is human: the genesis of human aneuploidy. Nat Rev Genet 2(4):280-291.
Heffner LJ. 2004. Advanced maternal age--how old is too old? N Engl J Med 351(19):1927-1929.
Hutchison CA, 3rd, Newbold JE, Potter SS, Edgell MH. 1974. Maternal inheritance of mammalian mitochondrial DNA. Nature 251(5475):536-538.
Jansen RP, de Boer K. 1998. The bottleneck: mitochondrial imperatives in oogenesis and ovarian follicular fate. Mol Cell Endocrinol 145(1-2):81-88.
Jazin EE, Cavelier L, Eriksson I, Oreland L, Gyllensten U. 1996. Human brain contains high levels of heteroplasmy in the noncoding regions of mitochondrial DNA. Proc Natl Acad Sci U S A 93(22):12382-12387.
Johns DR, Rutledge SL, Stine OC, Hurko O. 1989. Directly repeated sequences associated with pathogenic mitochondrial DNA deletions. Proc Natl Acad Sci U S A 86(20):8059-8062.
Judge S, Jang YM, Smith A, Hagen T, Leeuwenburgh C. 2005. Age-associated increases in oxidative stress and antioxidant enzyme activities in cardiac interfibrillar mitochondria: implications for the mitochondrial theory of aging. Faseb J 19(3):419-421.
Kaneda H, Hayashi J, Takahama S, Taya C, Lindahl KF, Yonekawa H. 1995. Elimination of paternal mitochondrial DNA in intraspecific crosses during early mouse embryogenesis. Proc Natl Acad Sci U S A 92(10):4542-4546.
Keefe DL, Niven-Fairchild T, Powell S, Buradagunta S. 1995. Mitochondrial deoxyribonucleic acid deletions in oocytes and reproductive aging in women. Fertil Steril 64(3):577-583.
Larsson NG, Wang J, Wilhelmsson H, Oldfors A, Rustin P, Lewandoski M, Barsh GS, Clayton DA. 1998. Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. Nat Genet 18(3):231-236.
Lee HC, Wei YH. 2001. Mitochondrial alterations, cellular response to oxidative stress and defective degradation of proteins in aging. Biogerontology 2(4):231-244.
Liu L, Keefe DL. 2002. Ageing-associated aberration in meiosis of oocytes from senescence-accelerated mice. Hum Reprod 17(10):2678-2685.

Long SE, Williams CV. 1982. A comparison of the chromosome complement of inner cell mass and trophoblast cells in day-10 pig embryos. J Reprod Fertil 66(2):645-648.
Lowder MQ, Mueller PO. 1998. Dental embryology, anatomy, development, and aging. Vet Clin North Am Equine Pract 14(2):227-245, v.
Magnusson C, Hillensjo T, Tsafriri A, Hultborn R, Ahren K. 1977. Oxygen consumption of maturing rat oocytes. Biol Reprod 17(1):9-15.
Magnusson C, LeMaire WJ. 1981. A gonadotrophin-releasing hormone agonist stimulates oxygen consumption and maturation of follicle-enclosed rat oocytes in vitro. Acta Physiol Scand 111(3):377-379.
McFeely RA. 1967. Chromosome abnormalities in early embryos of the pig. J Reprod Fertil 13(3):579-581.
Melov S, Shoffner JM, Kaufman A, Wallace DC. 1995. Marked increase in the number and variety of mitochondrial DNA rearrangements in aging human skeletal muscle. Nucleic Acids Res 23(20):4122-4126.
Menken J, Trussell J, Larsen U. 1986. Age and infertility. Science 233(4771):1389-1394.
Morris LH, Allen WR. 2002. Reproductive efficiency of intensively managed Thoroughbred mares in Newmarket. Equine Vet J 34(1):51-60.
Muller-Hocker J, Schafer S, Weis S, Munscher C, Strowitzki T. 1996. Morphological-cytochemical and molecular genetic analyses of mitochondria in isolated human oocytes in the reproductive age. Mol Hum Reprod 2(12):951-958.
Munne S, Alikani M, Tomkin G, Grifo J, Cohen J. 1995. Embryo morphology, developmental rates, and maternal age are correlated with chromosome abnormalities. Fertil Steril 64(2):382-391.
Munne S, Weier HU, Grifo J, Cohen J. 1994. Chromosome mosaicism in human embryos. Biol Reprod 51(3):373-379.
Murray JD, Moran C, Boland MP, Nancarrow CD, Sutton R, Hoskinson RM, Scaramuzzi RJ. 1986. Polyploid cells in blastocysts and early fetuses from Australian Merino sheep. J Reprod Fertil 78(2):439-446.
Muylle S, Simoens P, Lauwers H. 1996. Ageing horses by an examination of their incisor teeth: an (im)possible task? Vet Rec 138(13):295-301.
Nagley P, Wei YH. 1998. Ageing and mammalian mitochondrial genetics. Trends Genet 14(12):513-517.
Nybo Andersen AM, Wohlfahrt J, Christens P, Olsen J, Melbye M. 2000. Maternal age and fetal loss: population based register linkage study. Bmj 320(7251):1708-1712.
Pellestor F, Anahory T, Hamamah S. 2005. Effect of maternal age on the frequency of cytogenetic abnormalities in human oocytes. Cytogenet Genome Res 111(3-4):206-212.
Pellestor F, Andreo B, Arnal F, Humeau C, Demaille J. 2003. Maternal aging and chromosomal abnormalities: new data drawn from in vitro unfertilized human oocytes. Hum Genet 112(2):195-203.
Piko L, Taylor KD. 1987. Amounts of mitochondrial DNA and abundance of some mitochondrial gene transcripts in early mouse embryos. Dev Biol 123(2):364-374.
Rambags BPB, Krijtenburg PJ, Drie HF, Lazzari G, Galli C, Pearson PL, Colenbrander B, Stout TAE. 2005. Numerical chromosomal abnormalities in equine embryos produced in vivo and in vitro. Mol Reprod Dev 72(1):77-87.
Reynier P, May-Panloup P, Chretien MF, Morgan CJ, Jean M, Savagner F, Barriere P, Malthiery Y. 2001. Mitochondrial DNA content affects the fertilizability of human oocytes. Mol Hum Reprod 7(5):425-429.
Sastre J, Pallardo FV, Vina J. 2000. Mitochondrial oxidative stress plays a key role in aging and apoptosis. IUBMB Life 49(5):427-435.
Sauer MV. 1997. Infertility and early pregnancy loss is largely due to oocyte aging, not uterine senescence, as demonstrated by oocyte donation. Ann N Y Acad Sci 828:166-174.
Scheffler IE. 2000. A century of mitochondrial research: achievements and perspectives. Mitochondrion 1(1):3-31.
Schon EA, Rizzuto R, Moraes CT, Nakase H, Zeviani M, DiMauro S. 1989. A direct repeat is a hotspot for large-scale deletion of human mitochondrial DNA. Science 244(4902):346-349.
Shigenaga MK, Hagen TM, Ames BN. 1994. Oxidative damage and mitochondrial decay in aging. Proc Natl Acad Sci U S A 91(23):10771-10778.
Shoffner JM, Lott MT, Voljavec AS, Soueidan SA, Costigan DA, Wallace DC. 1989. Spontaneous Kearns-Sayre/chronic external ophthalmoplegia plus syndrome associated with a mitochondrial DNA deletion: a slip-replication model and metabolic therapy. Proc Natl Acad Sci U S A 86(20):7952-7956.
Steuerwald N, Barritt JA, Adler R, Malter H, Schimmel T, Cohen J, Brenner CA. 2000. Quantification of mtDNA in single oocytes, polar bodies and subcellular components by real-time rapid cycle fluorescence monitored PCR. Zygote 8(3):209-215.

Steuerwald N, Cohen J, Herrera RJ, Sandalinas M, Brenner CA. 2001. Association between spindle assembly checkpoint expression and maternal age in human oocytes. Mol Hum Reprod 7(1):49-55.

Sutovsky P, Moreno RD, Ramalho-Santos J, Dominko T, Simerly C, Schatten G. 1999. Ubiquitin tag for sperm mitochondria. Nature 402(6760):371-372.

Sutton ML, Cetica PD, Beconi MT, Kind KL, Gilchrist RB, Thompson JG. 2003. Influence of oocyte-secreted factors and culture duration on the metabolic activity of bovine cumulus cell complexes. Reproduction 126(1):27-34.

Takeuchi T, Neri QV, Katagiri Y, Rosenwaks Z, Palermo GD. 2005. Effect of treating induced mitochondrial damage on embryonic development and epigenesis. Biol Reprod 72(3):584-592.

Takeyama N, Matsuo N, Tanaka T. 1993. Oxidative damage to mitochondria is mediated by the Ca(2+)-dependent inner-membrane permeability transition. Biochem J 294 ( Pt 3):719-725.

te Velde ER, Pearson PL. 2002. The variability of female reproductive ageing. Hum Reprod Update 8(2):141-154.

Templeton A, Morris JK, Parslow W. 1996. Factors that affect outcome of in-vitro fertilisation treatment. Lancet 348(9039):1402-1406.

Thouas GA, Trounson AO, Jones GM. 2005. Effect of female age on mouse oocyte developmental competence following mitochondrial injury. Biol Reprod 73(2):366-373.

Thouas GA, Trounson AO, Wolvetang EJ, Jones GM. 2004. Mitochondrial dysfunction in mouse oocytes results in preimplantation embryo arrest in vitro. Biol Reprod 71(6):1936-1942.

Thundathil J, Filion F, Smith LC. 2005. Molecular control of mitochondrial function in preimplantation mouse embryos. Mol Reprod Dev 71(4):405-413.

Tilly JL. 2001. Commuting the death sentence: how oocytes strive to survive. Nat Rev Mol Cell Biol 2(11):838-848.

Tremoleda JL, Schoevers EJ, Stout TAE, Colenbrander B, Bevers MM. 2001. Organisation of the cytoskeleton during in vitro maturation of horse oocytes. Mol Reprod Dev 60(2):260-269.

Van Blerkom J, Davis PW, Lee J. 1995. ATP content of human oocytes and developmental potential and outcome after in-vitro fertilization and embryo transfer. Hum Reprod 10(2):415-424.

Van Tuyle GC, Gudikote JP, Hurt VR, Miller BB, Moore CA. 1996. Multiple, large deletions in rat mitochondrial DNA: evidence for a major hot spot. Mutat Res 349(1):95-107.

Viuff D, Rickords L, Offenberg H, Hyttel P, Avery B, Greve T, Olsaker I, Williams JL, Callesen H, Thomsen PD. 1999. A high proportion of bovine blastocysts produced in vitro are mixoploid. Biol Reprod 60(6):1273-1278.

Wallace DC. 1994. Mitochondrial DNA sequence variation in human evolution and disease. Proc Natl Acad Sci U S A 91(19):8739-8746.

Wallace DC, Ye JH, Neckelmann SN, Singh G, Webster KA, Greenberg BD. 1987. Sequence analysis of cDNAs for the human and bovine ATP synthase beta subunit: mitochondrial DNA genes sustain seventeen times more mutations. Curr Genet 12(2):81-90.

Zeilmaker GH, Verhamme CM. 1974. Observations on rat oocyte maturation in vitro: morphology and energy requirements. Biol Reprod 11(2):145-152.