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Reproductive competency and mitochondrial variation in aged Syrian hamster oocytes

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Abstract. The hamster is a useful model of human reproductive biology because its oocytes are similar to those in humans in terms of size and structural stability. In the present study we evaluated fecundity rate, ovarian follicular numbers, ova production, mitochondrial number, structure and function, and cytoplasmic lamellae (CL) in young (2–4 months) and old (12–18 months) Syrian hamsters (*Mesocricetus auratus*). Young hamsters had higher fertilisation rates and larger litters than old hamsters (100 vs 50% and 9.3 ± 0.6 vs 5.5 ± 0.6, respectively). Ovarian tissue from superovulated animals showed a 46% decrease in preantral follicles in old versus young hamsters. There was a 39% reduction in MII oocyte number in old versus young hamsters. Young ova had no collapsed CL, whereas old ova were replete with areas of collapsed, non-luminal CL. Eighty-nine per cent of young ova were expanded against the zona pellucida with a clear indentation at the polar body, compared with 58.64% for old ova; the remaining old ova had increased perivitelline space with no polar body indentation. Higher reactive oxygen species levels and lower mitochondrial membrane potentials were seen in ova from old versus young hamsters. A significant decrease in mitochondrial number (36%) and lower frequency of clear mitochondria (31%) were observed in MII oocytes from old versus young hamster. In conclusion, the results of the present study support the theory of oocyte depletion during mammalian aging, and suggest that morphological changes of mitochondria and CL in oocytes may be contributing factors in the age-related decline in fertility rates.

Additional keywords: aging, lamellae, mitochondria, organelle structure, reactive oxygen species (ROS).

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Introduction

Age-related decline in fertility begins at the age of 30 years in humans and falls steadily until menopause (Alviggi *et al.* 2009). The quantity and quality of ovarian follicles with their associated ova are primary contributing factors in the reproductive aging process. A decline in mitochondrial energy metabolism and enhanced oxidative stress are also important contributors to aging (Lee and Wei 2012). Mitochondria are considered essential organelles in the aging process of ovarian follicles (Jansen and Burton 2004) and are involved in specialised processes, including signal transduction, calcium homeostasis and oxygen sensing, in addition to ATP production (Bunn and Poyton 1996; Pozzan *et al.* 2000; Quintero *et al.* 2006; Van Blerkom 2008). Mitochondrial proliferation occurs in parallel with increased cellular metabolism and increased ATP needs of the oocyte, which are generally associated with increasing cytoplasmic volume (Jansen and Burton 2004). Age-related functional and morphological changes have been well documented in oocytes and oocyte mitochondria of humans, mice and rats. These changes include increased mitochondrial matrix

density and change of distribution (de Bruin *et al.* 2004; Nagai *et al.* 2006), increased reactive oxygen species (ROS) production (Tatone *et al.* 2011), increased mitochondrial (mt) DNA mutations, decreased mitochondrial membrane potential ($\Delta\Psi_m$; Zeng *et al.* 2009) and decreased mitochondria–smooth endoplasmic reticulum (M-SER) and increased mitochondria–vesicle aggregates (Bianchi *et al.* 2015). However, studies on the age-related changes of oocytes rarely include hamsters, and such age-related mitochondrial changes in hamster oocytes have not been studied to date.

Hamsters are an excellent animal model for the study of oocytes for several reasons: (1) the hamster ovum (120 μm) is closer in size to the human ovum (150–200 μm) than the mouse ovum (80–100 μm; Griffin *et al.* 2006); (2) female hamsters show a regular and predictable 4-day oestrous cycle (Hendry *et al.* 2002); (3) large numbers of oocytes (30–60) per young female can be obtained from a single superovulation; (4) the optimal light cycle of the hamster is 14 h light and 10 h dark, which is closer to that of humans (optimal 9 h sleep) than to the12 h light–12 h dark cycle for other rodents, such as the mouse; (5) the hamster is relatively free from spontaneous disease (Fox 1985); and (6) the short 16-day gestation period and 4-day oestrus cycle make the Syrian hamster a more efficacious model than large animals to manipulate for reproductive research. Mizoguchi and Dukelow (1981) reported that, much like the situation in humans, fertilisation rates in hamsters decrease with age and suggested that defective ova were one of the major factors resulting in increased preimplantation loss in aged hamsters.

The primary aim of the present study was to investigate oocyte depletion during aging and to identify microscopic variations of mitochondria in oocytes from young and old hamsters.

Materials and methods

Animals and reagents

Female golden Syrian hamsters (HsdHan; AURA, Harlan Laboratories) were grouped into a young hamster group (2–4 months) and an old hamster group (12–16 months) according to the classification of Parkening *et al.* (1982). The hamsters were housed in a temperature- and light-controlled room. All experimental protocols and animal handling procedures were conducted in accordance with the National Research Council (NRC) publication Guide for Care and Use of Laboratory Animals (National Academy of Science 1996), as reviewed and approved by the Old Dominion University Institutional Animal Care and Use Committee (IACUC; Protocol no. 10-035).

Fecundity rate counting

In order to confirm the higher fecundity rate of young versus old hamsters, eight young female hamsters and eight old female hamsters were mated with a group of male hamsters of the same age.

Superovulation

Old female hamsters selected at random with regard to oestrous cycle were induced to superovulate by intraperitoneal (i.p.) injection of 5 IU per 50 g hamster weight; 30 IU per old hamster and 20 IU per young hamster pregnant mare's serum gonadotropin (PMSG; Sigma-Aldrich) followed 54 h later by human chorionic gonadotropin (hCG; Sigma-Aldrich) 30 IU per old hamster and 20 IU per young hamster. Superovulated females were killed by cervical dislocation after anaesthesia via sodium pentobarbital (Sheris) approximately 17 h after hCG injection for MII oocyte retrieval.

Histological preparation and evaluation of ovarian follicles

In order to evaluate and compare preantral follicles in young and old ovarian tissue, ovaries were collected from superovulated Syrian hamsters and processed in 10% buffered formalin through a series of dehydration steps, paraffin embedded, serial sectioned (8 μm) with a Leica RM2265 microtome, mounted on slides and stained with haematoxylin and eosin (HE) for preantral follicle counts. Preantral follicles of hamsters contained a germinal vesicle and either a single layer of squamous epithelial cells or one to three layers of cuboidal epithelial (granulosa) cells with follicular antra according to previous descriptions (Griffin *et al.* 2006; Araújo *et al.* 2014).

Collection of superovulated MII oocytes

Oviducts excised from anaesthetised hamsters were placed in sterile 35-mm polystyrene Nunclon culture dishes with 3 mL of M199TE medium as described by Yamauchi *et al.* (2002). Under a Zeiss dissecting stereomicroscope at ×20 magnification, watchmaker forceps were used to slide the fimbriae onto a 26-gauge sterile needle attached to a 1-mL sterile syringe. Cumulus–oocyte complexes (COCs) were irrigated from the oviducts with medium expressed from the syringe with moderate force. COCs from individual hamsters were placed in a drop of 1% hyaluronidase (Sigma-Aldrich) under mineral oil and evaluated continuously for cumulus cells dissociating from the oocytes. Oocytes that were close to being cell free were then picked up with a hand-pulled glass micropipette (~100 μm lumen inner diameter) and washed three times through hyaluronidase-free M199TE medium with vigorous pipetting to remove all cumulus cells. Normal MII stage eggs (judged by minimal perivitelline space (PVS) with one small polar body pressed against the oolemma) were evaluated at ×32 magnification and collected from the fourth wash droplet to a Petri dish with M199TE medium for the experimental protocols.

Evaluating oocyte perivitelline space

All ovulated oocytes were counted. Those oocytes that were expanded tightly against the zona pellucida (minimal PVS) with a clear indentation at the polar body were judged to be healthy oocytes (Graham *et al.* 1994; Xia 1997). Oocytes with a wide PVS were judged poor in quality and discarded.

Fluorescence labelling of mitochondria with JC-1 and confocal microscopy

The potential-sensitive fluorescence dye 5,5′,6,6′-tetrachloro-1,1,3,3′-tetraethylbenzimdazoylcarbocyanine iodide (JC-1; Invitrogen), was used to measure $\Delta\Psi_m$ and thereby to assess the activity of the mitochondria. The process was performed as described previously (Thouas *et al.* 2004; Zeng *et al.* 2009).

Low-polarised mitochondria with $\Delta\Psi_m$ <100 mV (negative inside) generally show green fluorescence under the confocal microscope because of accumulation of the monomer form of JC-1 within the organelle. Highly polarised mitochondria ($\Delta\Psi_m$ >140 mV) are indicated by red fluorescence because of JC-1 aggregate formation. In the present study, retrieved oocytes were incubated in the JC-1 working solution (1 μM) for 30 min at 37°C. Fluorescence was evaluated under a Zeiss LSM-510 confocal laser scanning microscope equipped with a Kr–Ar laser to produce an excitation wavelength of 488 nm; a 530 nm dichroic mirror was used for the emission wavelength. A Z-section scan at 5-μm intervals through the centre of the oocyte was used to analyse fluorescence intensity, as described by Van Blerkom *et al.* (2002).

Quantification of ROS in oocytes with MitoTracker Red CM-H2XRos

Intracellular ROS was quantified using MitoTracker Red CM-H2XRos (MRR, Invitrogen). MRR is oxidised by ROS, mainly by superoxide and hydrogen peroxide, and trapped in mitochondria by its chloromethyl moiety (Kweon *et al.* 2001). The reduced form of this dye may also be used to determine intracellular levels of ROS (Park *et al.* 2006). Experiments were performed in a similar way to those described for JC-1 staining above. The final working solution was 1 μM MRR in M199TE buffer. Fluorescence emitted from MRR passed through a 600 long pass (LP) emission filter. Images of JC-1 and MRR fluorescence were processed and the fluorescence intensity in the oocytes was measured using MetaMorph 7.5 (JH Technologies).

Oocyte preparation for transmission electron microscopy

The preparation of oocytes for transmission electron microscopy (TEM) used a modification of procedures described by Britton *et al.* (1991). Oocytes were collected as described above and were fixed in 2.5% glutaraldehyde (GA) in 0.1 M phosphate buffer (PB) for 4 h at 4°C. Subsequently, oocytes were washed twice in 0.1 M PB and then once in 10% bovine serum albumin (BSA) in Dulbecco's phosphate-buffered saline (PBS) before being transferred to a Beem capsule (flat bottom Size 3, Electron Microscopy Sciences) containing 1 drop of 10% BSA. Following 30 min rest, capsules were spun in a swinging bucket centrifuge at 1800*g* for 15 min at room temperature. Three drops of GA were carefully overlaid on the surface of the BSA to avoid mixing. The capsules were centrifuged horizontally 1800*g* for 1 h at room temperature and subsequently filled with GA and refrigerated at 4°C overnight. The following morning, the GA was poured off and the fixed protein–oocyte mould was transferred from the Beem capsule to a 1-mL vial. The mould was washed twice in 0.1 M PB and post-fixed with osmium tetroxide (1% osmium tetroxide in 0.1 M PBS) for 2 h and then washed twice in PB. The mould was dehydrated in ethanol gradients and infiltrated with pure propylene oxide twice for 15 min each time. The mould was then infiltrated in 1 : 1 EMBed 812 (Electron Microscopy Sciences) mixture and propylene oxide for 1 h, followed by pure EMBed 812 for 1 h. The EMBed 812 mixture was made from an EMBed 812 kit (Electron Microscopy Sciences) with 20 mL EMBed 812, 9 mL dodecenyl succinic anhydride (DDSA), 12 mL methyl-5-norbornene-2,3-dicarboxylic anhydride (NMA) and 0.75 mL dimethylaminomethyl phenol (DMP-30). The mould was aligned into a Beem capsule filled with EMBed 812 mixture and polymerised in a 60°C oven for 48 h. The samples were cut with an RMC-MT2C ultra microtome (RMC Boeckeler). Thin sections were collected on G200-Cu grids (Electron Microscopy Sciences), stained sequentially with uranyl acetate and lead citrate and examined on a JEM-1200EXI electron microscope (Jeol USA, Inc.). The analysis was performed with ImageJ with plug-ins downloaded from the National Institute of Health following the method described by Weibel *et al.* (1966).

Statistical analysis

Data were analysed using SPSS version 18.0 (SPSS Inc.). The PVS of oocytes and ratio of dark to light mitochondria were evaluated with Student's *t*-test after transformation to arcsine. Other results were compared using Student's *t*-test. Results are presented as the mean ± s.e.m., and two-tailed $P \leq 0.05$ was considered significant.

Results

Decline in fecundity rate from young to old hamsters

Fertilisation rates were found to be 100% for young hamsters and 50% for old hamsters ($n = 8$), with the average numbers of pups being 9.3 ± 0.6 and 5.5 ± 0.6 for young and old hamsters respectively, suggesting that young hamsters have higher fertilisation rates and larger litters than old hamsters. These reductions in fecundity and litter size for the aging hamster are in agreement with the decline in fertility seen in other species (David *et al.* 1975; Giesel 1979).

Evaluation of ovarian tissue and number of superovulated oocytes

Ovaries collected from old and young hamsters showed a 46% decrease in the number of preantral follicles in old compared with young hamster ovaries (48.3 ± 3.5 vs 90.0 ± 12.5; Fig. 1).

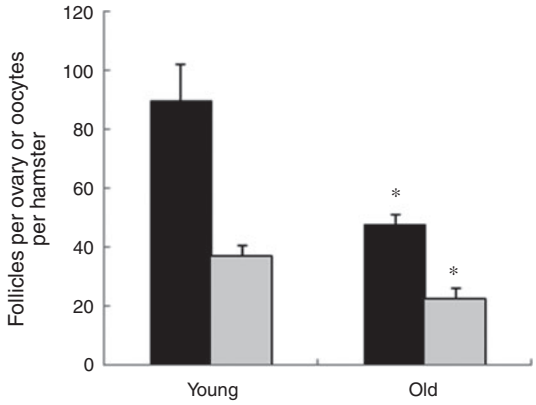

Fig. 1. Number of ovarian follicles and superovulated oocytes. Black columns show the number of follicles per ovary (young ovaries, $n = 6$; old ovaries, $n = 4$); grey columns show the number of superovulated oocytes per hamster ($n = 20$ in each group). Data are the mean ± s.e.m. *$P < 0.01$.

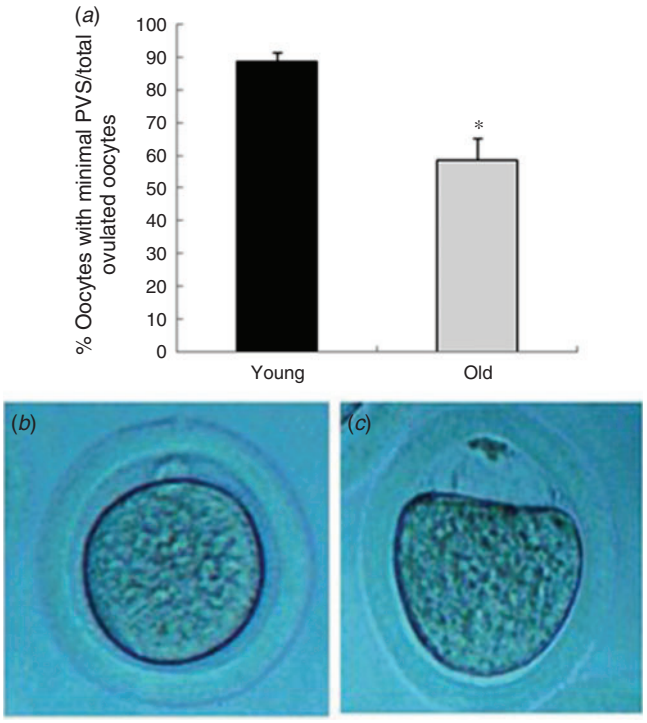

Fig. 2. Morphological differences between oocytes from young and old hamsters. (*a*) Young hamsters have a significantly higher percentage of 'healthy-appearing' oocytes (88.68%; $n = 14$) than old hamsters (58.64%; $n = 17$). Data are the mean ± s.e.m. of 522 oocytes from 14 young hamsters and 322 oocytes from 17 old hamsters. *$P < 0.01$ (*b*, *c*) Representative micro photographs showing that young oocytes (*b*) were expanded against the zona pellucida with a minimal perivitelline space (PVS) and a clear indentation at the polar body, whereas old oocytes (*c*) had wide PVS with no indentation from the polar body.

A 39% reduction in the number of ovulated MII oocytes was observed in superovulated old versus young hamsters (22.6 ± 3.6 vs 37.1 ± 3.7 respectively; $P < 0.05$, Student's *t*-test; Fig. 1). These results indicate an age-related decline in the number of preantral follicles and ovulated MII oocytes in hamsters.

Evaluating the perivitelline space of oocytes

Of 522 oocytes retrieved from 14 young hamsters, 403 were predominantly expanded against the zona pellucida (minimal PVS) with a clear indentation at the polar body (Fig. 2), thus qualifying these as 'healthy' oocytes (Xia 1997; Rienzi *et al.* 2008). However, only 189 of 322 oocytes from 17 old hamsters could be described in this way. The remaining 'non-healthy' old oocytes had a wide PVS with no indentation from the polar body. These data indicate that young hamsters produce a higher percentage of morphologically healthy-appearing oocytes than old hamsters (88.7 ± 2.7% vs 58.6 ± 6.6% respectively). This result reflects a significant, age-related, 33.9% decrease of healthy oocytes in old versus young hamsters ($P < 0.001$, Student's *t*-test with arcsine transformation).

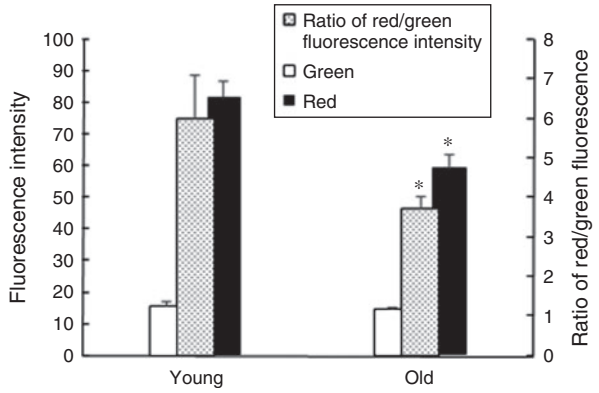

Fig. 3. Red and green fluorescent signals in JC-1-stained oocytes, and the ratio of red : green fluorescence in MII oocytes from young ($n = 30$) and old ($n = 29$) hamsters. Data are the mean ± s.e.m. of independent experiments for six female golden hamsters in each group. *$P < 0.01$.

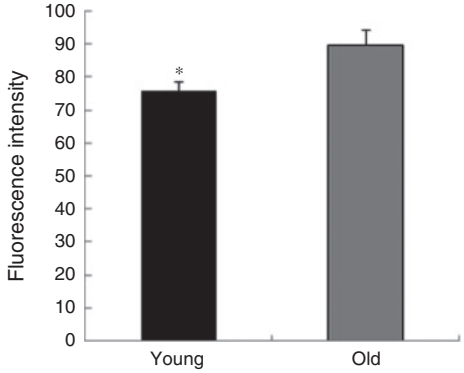

Fig. 4. Fluorescent signals of oocytes stained with MitoTracker Red CM-H2XRos (MRR). Fluorescence intensity significantly higher in oocytes from old than young hamsters ($P < 0.05$; $n = 30$). The independent experiments represented 6 female golden hamsters in each groups. Data are the mean ± s.e.m. of independent experiments for six female golden hamsters in each group. *$P < 0.01$.

$\Delta\Psi_m$ in MII oocytes from Young and Old Hamsters

The potential-sensitive fluorescent dye JC-1 was used to measure $\Delta\Psi_m$. As seen in Fig. 3, the red fluorescence intensity of oocytes from young hamsters was significantly higher than that from old hamsters (81.9 ± 4.7 vs 59.5 ± 3.9 respectively; $n = 30$; $P < 0.01$, Student's *t*-test). The young oocytes had a significantly higher ratio of JC-1 red/green fluorescence intensity than old oocytes (6.0 ± 2.8 vs 3.7 ± 1.0 respectively; $P < 0.01$, Student's *t*-test; Fig. 3). This indicates that oocytes from young hamsters contain more mitochondria (~62% more) with higher $\Delta\Psi_m$ than oocytes from old hamsters.

ROS levels in MII oocytes from young and old hamsters

MRR was used to evaluate changes in ROS levels in young and old oocytes. The fluorescence in oocytes from old hamsters (90.9 ± 4.5) incubated with MRR showed a significant increase ($P < 0.05$; $n = 30$) in ROS production compared with young hamsters (75.7 ± 3.1; Fig. 4).

Mitochondrial morphological differences between young and old hamster oocytes by TEM

Fig. 5 shows examples of mitochondrial morphological differences between MII oocytes from young and old hamsters, with representative images from six young (Fig. 5*a*) and six old (Fig. 5*b*) female hamsters. There was a significant 36% decrease in mitochondrial number in aged versus young hamster oocytes (75.5 ± 2.8 vs 55.4 ± 2.8 per $100\,\mu m^2$ respectively; $P < 0.01$, Student's *t*-test with arcsine transformation; Fig. 5*g*).

Both young and old oocytes contained clear mitochondria with distinct cristae, as well as some dark mitochondria with large amounts of electron-dense areas (Fig. 5*c*, *d*), but old oocytes, on average, contained a significantly lower (31%) frequency of clear mitochondria than young oocytes ($38.6 \pm 1.4\%$ vs $55.6 \pm 2.1\%$ respectively; $P < 0.01$; Fig. 5*h*). At higher magnification, collapsed cytoplasmic lamellae were seen within old oocytes (Fig. 5*f*), but not in young oocytes (Fig. 5*e*).

Discussion

The number and functionality of oocyte and embryonic mitochondria have been recognised as major determinants of both pregnancy potential and successful progression through fetal development and birth. Decreased fecundity with increasing age has been observed in several mammalian species (Giesel 1979). Because structural and functional changes have been shown to occur in aged mammalian mitochondria, several groups have investigated the relationship between aging, mitochondrial function and fertility (Jansen and Burton 2004; Thouas *et al.* 2004; Van Blerkom 2008; Zeng *et al.* 2009). However, even though the hamster has become a very useful animal model for reproduction studies, a review of the literature finds relatively few analyses of oocytes and mitochondrial structure and/or function in this animal. Mizoguchi and Dukelow (1981) reported that defective oocytes with chromosomal abnormality represent one major factor leading to increased preimplantation loss in the aging hamster. We have reported previously decreased ATP and mtDNA number in individual oocytes from old hamsters (Simsek-Duran *et al.* 2013). In the present study, we found morphological differences between young and old hamster oocytes by light, transmission electron and fluorescence microscopy. Old hamsters produced a significantly lower percentage (33.87% lower) of healthy-appearing oocytes with minimal PVS compared with those in young hamsters. A large PVS has been related to lower fertilisation rates in humans (Rienzi *et al.* 2008). This is in agreement with our finding of lower fecundity rates in old compared with young female hamsters, which is in agreement with an earlier observation in hamsters (Mizoguchi and Dukelow 1981).

Oocyte quality has been associated with the size of the mitochondrial population (Reynier *et al.* 2001). Oocyte energy is derived primarily from mitochondrial ATP, so healthy mitochondria are needed in sufficient numbers to maintain the ATP levels necessary for oocyte development and fertilisation. In a previous study, we showed that mtDNA number, and by extension the number of mitochondria, decreased significantly in MII stage oocytes from old compared with young hamsters (Simsek-Duran *et al.* 2013).

Ultrastructural evaluation of mitochondria further confirmed the significant difference in mitochondrial number in oocytes from young and old hamsters. The TEM analysis in the present study showed that oocytes from young hamsters contained, on average, 36% more mitochondria than those from old hamsters. Mitochondrial quantity and quality are both important for normal oocyte development and fertilisation.

$\Delta\Psi_m$ is another important index of mitochondrial function. A decrease in $\Delta\Psi_m$ reflects mitochondrial dysfunction and is related to a series of mitochondrial functional changes such as increased ROS production, decreased ATP levels and irregular calcium regulation (Van Blerkom 2011; Bellanti *et al.* 2013; Duicu *et al.* 2013). Based on the increased red fluorescence in JC-1-stained oocytes, mitochondria in young oocytes have a higher polarisation of their inner membrane than old oocytes. To avoid bias from the difference in mitochondrial quantity between young and old oocytes, we used the ratio of red to green fluorescence intensity to reliably describe this change. The ratio of red : green fluorescence intensity was significantly lower in oocytes from old compared with young hamsters. According to the characteristics of JC-1, this implies that a greater percentage of the mitochondria in young oocytes maintain a higher polarisation than those in old oocytes. Because the energy stored in the mitochondrial membrane potential drives ATP synthesis, reduced polarisation in mitochondria from old oocytes would lead to reduced ATP production. It remains to be determined whether decreased $\Delta\Psi_m$ is an inducer or a subsequent event of the apoptotic pathway (Klamt and Shacter 2005; Boren and Brindle 2012); in either case, the decrease in $\Delta\Psi_m$ indicates the possibility of increased apoptosis occurring in older oocytes. This decrease in $\Delta\Psi_m$ may contribute to the poor development of old oocytes and low fecundity of old hamsters. ROS levels were significantly higher in oocytes from old than young hamsters, suggesting that there is an accumulation of defective mitochondria with higher ROS production in oocytes from old hamsters. Elizur *et al.* (2014) found that high-quality embryos were derived from follicles with lower ROS levels, whereas poor-quality embryos developed from follicles with higher ROS levels. Increased intracellular ROS has also been shown to cause the fragmentations seen in porcine MII oocytes during aging (Tang *et al.* 2013). Conflicting reports have been published on changes in $\Delta\Psi_m$ and ROS generation (Brookes 2005). Some studies show mitochondrial uncoupling as a cytoprotective strategy to limit ROS generation, whereas others think the collapse in $\Delta\Psi_m$ induces ROS generation (Roy *et al.* 2004; Speakman *et al.* 2004). The relationship between ROS production and apoptosis has been well documented and we propose that the increase in ROS production demonstrated herein also contributes to mitochondrial dysfunction.

Evaluation of morphological changes in mitochondrial ultrastructure indicated that old oocytes manifested a higher frequency of dark mitochondria with few or absent distinct cristae. These observations of hamster oocyte morphology agree with the increase in cristae complexity and electron density seen in the mitochondrial matrix with both *in vivo* and *in vitro* human oocyte aging (Sathananthan 1997). Mitochondrial morphology changes with follicular development. It has been reported that the electron density of the mitochondrial matrix and the complexity

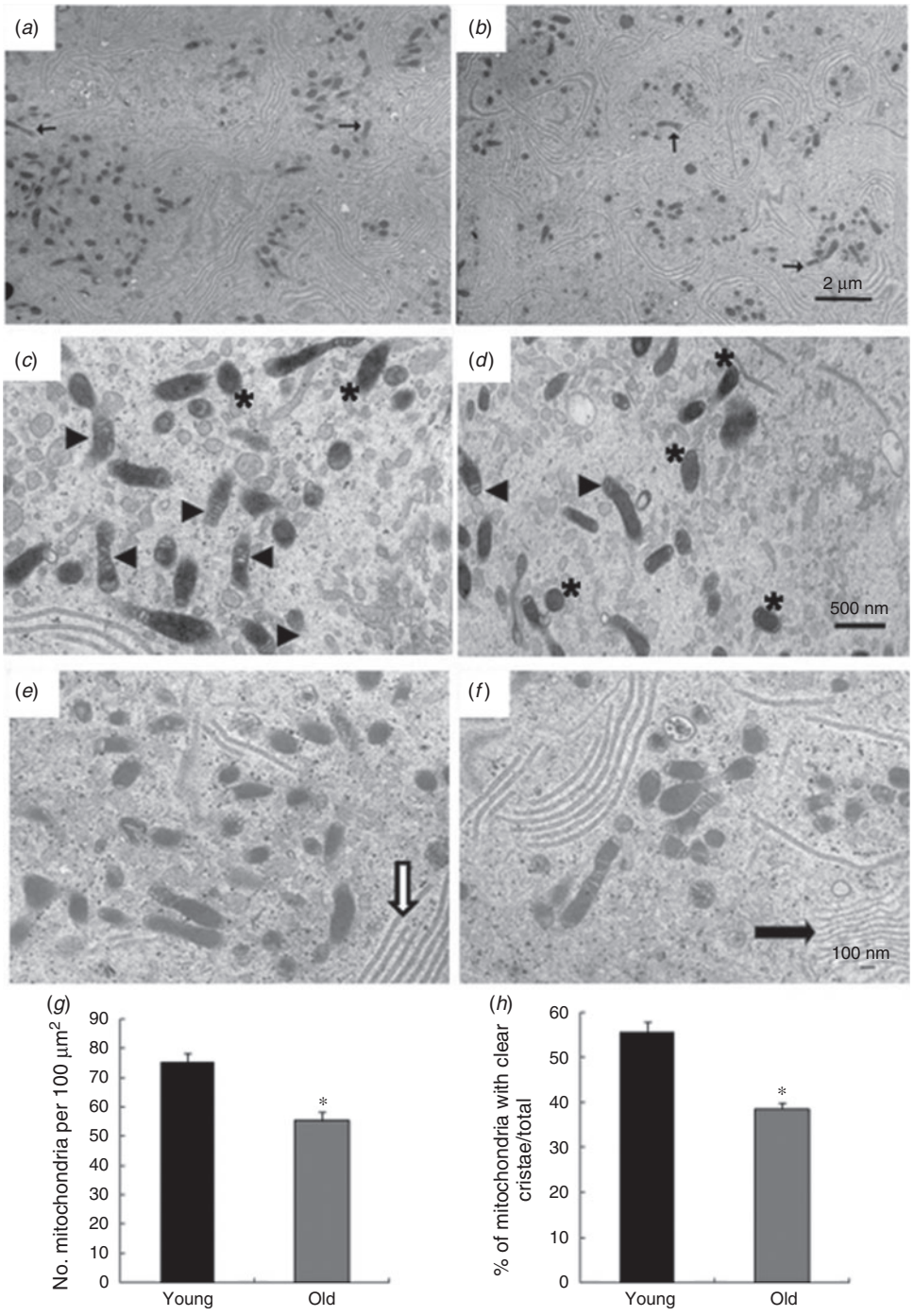

Fig. 5. Mitochondrial morphology of oocytes from (*a*, *c*, *e*) young and (*b*, *d*, *f*) old hamsters. (*a*, *b*) There was a significantly higher number of mitochondria (arrows) in oocytes from young (*a*) than old (*b*) hamsters ($P < 0.01$). (*c*, *d*) Note the dark mitochondria (asterisks) and mitochondria with clear cristae (arrowheads). All oocytes contain some dark mitochondria with large amounts of electron-dense areas, but old oocytes (*d*) exhibit a significantly higher frequency of dark mitochondria without distinct cristae ($P < 0.01$). (*e*, *f*) Collapsed cytoplasmic lamellae (filled arrows) and normal cytoplasmic lamellae (open arrows) in old (*f*) but not young (*e*) young oocytes. (*g*, *h*) Graphical representation of the significant 36% decrease in mitochondrial number in old versus young hamster oocytes (*g*) and the percentage of mitochondria with clear cristae (*h*). Old oocytes contained significantly (31%) fewer clear mitochondria with distinct cristae than young oocytes. Data are the mean ± s.e.m. of independent experiments for six female golden hamsters in each group. $*P < 0.05$.

of cristae increase in the rapidly enlarging oocyte until follicular antrum formation, declining thereafter (Weakley 1976; Weakley and James 1982). It has also been suggested that during differentiation of the embryo there is a gradual change from mitochondria with dense matrices to elongated mitochondria with less dense matrices and numerous cristae (Motta *et al.* 2000). Ishida *et al.* (1979) suggested that high electron density may be the result of the sequestration and storage of calcium. Therefore, the intensity of the electron density in the matrix of the aged hamster mitochondria seen in the present study may reflect, in part, the mature nature of the oocytes and the changing role of mitochondria in calcium regulation due to aging.

The density changes in mitochondria are intimately associated with the SER (Weakley and James 1982). The TEM results in the present study did not reveal changes in regular vesicle SER, but we observed some CL. CL are a conformational change in the SER (Banno and Kohno 1996) and collapsed forms of these structures were observed only in oocytes from old hamsters. A decrease in M-SER aggregates has been reported both during reproductive aging and in *in vitro* aged oocytes (Motta *et al.* 2000; Bianchi *et al.* 2015). The abnormalities of both CL and mitochondria together may indicate a dysfunction in the regulation of calcium concentration in the cytoplasm and mitochondrial matrix, because mitochondria and SER play important roles in regulating calcium concentration. Changes in cortical granules (CG) have also been associated with fertility differences in cows and mice (Ducibella *et al.* 1990; Båge *et al.* 2003; Bianchi *et al.* 2015), but we did not find morphological changes in CG between oocytes from old and young hamsters (F Li, W Ford, FJ Castora, RJ Swanson, unpubl. data).

In addition to showing differences in oocyte quality and quantity in young and old hamsters, the present study illustrates both mitochondrial dysfunctions and morphological changes in oocytes from old versus young hamsters. These parameters most likely contribute to lower fertility and fecundity rates through decreased ATP production, abnormal regulation of calcium concentration and transfer and increased levels of ROS, in addition to other mechanisms that have not yet been elucidated. The data we present herein (a reduction in fecundity, fertilisation rate and litter size with advanced age in the hamster correlating with increased levels of ROS, decreased mitochondrial membrane potential and a variety of morphologic changes) further support the conclusion that these molecular and morphological changes contribute to, if not determine, the age-related difficulties in mammalian conception, development to term and delivery of healthy offspring in the hamster. We believe that these molecular and morphological changes in the aged hamster oocyte mimic those of the human, making the hamster a preferred animal model for studying human fertilisation and embryonic development.

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References

Alviggi, C., Humaidan, P., Howles, C. M., Tredway, D., and Hillier, S. G. (2009). Biological versus chronological ovarian age: implications for assisted reproductive technology. *Reprod. Biol. Endocrinol.* **7**, 101. doi:10.1186/1477-7827-7-101

Araújo, V. R., Gastal, M. O., Figueiredo, J. R., and Gastal, E. L. (2014). *In vitro* culture of bovine preantral follicles: a review. *Reprod. Biol. Endocrinol.* **12**, 78–91. doi:10.1186/1477-7827-12-78

Båge, R., Petyim, S., Larsson, B., Hallap, T., Bergqvist, A. S., Gustafsson, H., and Rodriguez-Martinez, H. (2003). Oocyte competence in repeat-breeder heifers: effects of an optimized ovum pick-up schedule on expression of oestrus, follicular development and fertility. *Reprod. Fertil. Dev.* **15**, 115–123. doi:10.1071/RD02094

Banno, T., and Kohno, K. (1996). Conformational changes of smooth endoplasmic reticulum induced by brief anoxia in rat Purkinje cells. *J. Comp. Neurol.* **369**, 462–471. doi:10.1002/(SICI)1096-9861(19960603)369:3<462::AID-CNE10>3.0.CO;2-K

Bellanti, F., Romano, A. D., Giudetti, A. M., Rollo, T., Blonda, M., Tamborra, R., Vendemiale, G., and Serviddio, G. (2013). Many faces of mitochondrial uncoupling during age: damage or defense? *J. Gerontol. A Biol. Sci. Med. Sci.* **68**, 892–902. doi:10.1093/GERONA/GLS332

Bianchi, S., Macchiarelli, G., Micara, G., Linari, A., Boninsegna, C., Aragona, C., Rossi, G., Cecconi, S., and Nottola, S. A. (2015). Ultrastructural markers of quality are impaired in human metaphase II aged oocytes: a comparison between reproductive and *in vitro* aging. *J. Assist. Reprod. Genet.* **32**, 1343–1358. doi:10.1007/S10815-015-0552-9

Boren, J., and Brindle, K. M. (2012). Apoptosis-induced mitochondrial dysfunction causes cytoplasmic lipid droplet formation. *Cell Death Differ.* **19**, 1561–1570. doi:10.1038/CDD.2012.34

Britton, A. P., Moon, Y. S., and Yuen, B. H. (1991). A simple handling technique for mammalian oocytes and embryos during preparation for transmission electron microscopy. *J. Microsc.* **161**, 497–499. doi:10.1111/J.1365-2818.1991.TB03107.X

Brookes, P. S. (2005). Mitochondrial H(+) leak and ROS generation: an odd couple. *Free Radic. Biol. Med.* **38**, 12–23. doi:10.1016/J.FREERADBIOMED.2004.10.016

Bunn, H. F., and Poyton, R. O. (1996). Oxygen sensing and molecular adaptation to hypoxia. *Physiol. Rev.* **76**, 839–885.

David, J., Cohet, Y., and Foluillet, P. (1975). The variability between individuals as a measure of senescence: a study of the number of eggs laid and the percentage of hatched eggs in the case of *Drosophila melanogaster*. *Exp. Gerontol.* **10**, 17–25. doi:10.1016/0531-5565(75)90011-X

de Bruin, J. P., Dorland, M., Spek, E. R., Posthuma, G., van Haaften, M., Looman, C. W., and te Velde, E. R. (2004). Age-related changes in the ultrastructure of the resting follicle pool in human ovaries. *Biol. Reprod.* **70**, 419–424. doi:10.1095/BIOLREPROD.103.015784

Ducibella, T., Duffy, P., Reindollar, R., and Su, B. (1990). Changes in the distribution of mouse oocyte cortical granules and ability to undergo the cortical reaction during gonadotropin-stimulated meiotic maturation and aging *in vivo*. *Biol. Reprod.* **43**, 870–876. doi:10.1095/BIOLREPROD43.5.870

Duicu, O. M., Mirica, S. N., Gheorgheosu, D. E., Privistirescu, A. I., Fira-Mladinescu, O., and Muntean, D. M. (2013). Ageing-induced decrease in cardiac mitochondrial function in healthy rats. *Can. J. Physiol. Pharmacol.* **91**, 593–600. doi:10.1139/CJPP-2012-0422

Elizur, S. E., Lebovitz, O., Orvieto, R., Dor, J., and Zan-Bar, T. (2014). Reactive oxygen species in follicular fluid may serve as biochemical markers to determine ovarian aging and follicular metabolic age. *Gynecol. Endocrinol.* **30**, 705–707. doi:10.3109/09513590.2014.924100

Fox, J. G. (1985). Laboratory animal medicine. Changes and challenges. *Cornell Vet.* **75**, 159–170.

Giesel, J. T. (1979). Associations between age specific mortality and fecundity rates in mammals. *Exp. Gerontol.* **14**, 189–192. doi:10.1016/0531-5565(79)90018-4

Graham, J., Ford, T., and Rickwood, D. (1994). The preparation of subcellular organelles from mouse liver in self-generated gradients of iodixanol. *Anal. Biochem.* **220**, 367–373. doi:10.1006/ABIO.1994.1351

Griffin, J., Emery, B. R., Huang, I., Peterson, C. M., and Carrell, D. T. (2006). Comparative analysis of follicle morphology and oocyte diameter in four mammalian species (mouse, hamster, pig, and human). *J. Exp. Clin. Assist. Reprod.* **3**, 2. doi:10.1186/1743-1050-3-2

Hendry, W. J., 3rd, Sheehan, D. M., Khan, S. A., and May, J. V. (2002). Developing a laboratory animal model for perinatal endocrine disruption: the hamster chronicles. *Exp. Biol. Med. (Maywood)* **227**, 709–723.

Ishida, A., Mashima, H., and Tanaka, S. (1979). Intracellular distribution of calcium in cardiac muscles studied by electron microscope autoradiography. *Jpn. J. Physiol.* **29**, 37–48. doi:10.2170/JJPHYSIOL.29.37

Jansen, R. P., and Burton, G. J. (2004). Mitochondrial dysfunction in reproduction. *Mitochondrion* **4**, 577–600. doi:10.1016/J.MITO.2004.07.038

Klamt, F., and Shacter, E. (2005). Taurine chloramine, an oxidant derived from neutrophils, induces apoptosis in human B lymphoma cells through mitochondrial damage. *J. Biol. Chem.* **280**, 21 346–21 352. doi:10.1074/JBC.M501170200

Kweon, S. M., Kim, H. J., Lee, Z. W., Kim, S. J., Kim, S. I., Paik, S. G., and Ha, K. S. (2001). Real-time measurement of intracellular reactive oxygen species using Mito tracker orange (CMH2TMRos). *Biosci. Rep.* **21**, 341–352. doi:10.1023/A:1013290316939

Lee, H. C., and Wei, Y. H. (2012). Mitochondria and aging. *Adv. Exp. Med. Biol.* **942**, 311–327. doi:10.1007/978-94-007-2869-1_14

Mizoguchi, H., and Dukelow, W. R. (1981). Fertilizability of ova from young or old hamsters after spontaneous or induced ovulation. *Fertil. Steril.* **35**, 79–83. doi:10.1016/S0015-0282(16)45263-5

Motta, P. M., Nottola, S. A., Makabe, S., and Heyn, R. (2000). Mitochondrial morphology in human fetal and adult female germ cells. *Hum. Reprod.* **15**(Suppl. 2), 129–147. doi:10.1093/HUMREP/15.SUPPL_2.129

Nagai, S., Mabuchi, T., Hirata, S., Shoda, T., Kasai, T., Yokota, S., Shitara, H., Yonekawa, H., and Hoshi, K. (2006). Correlation of abnormal mitochondrial distribution in mouse oocytes with reduced developmental competence. *Tohoku J. Exp. Med.* **210**, 137–144. doi:10.1620/TJEM.210.137

National Academy of Science (1996) 'Guide for care and use of laboratory animals.' Available at http://www.nap.edu/catalog/5140/guide-for-the-care-and-use-of-laboratory-animals [Verified 23 May 2016]

Park, E. S., Gao, X., Chung, J. M., and Chung, K. (2006). Levels of mitochondrial reactive oxygen species increase in rat neuropathic spinal dorsal horn neurons. *Neurosci. Lett.* **391**, 108–111. doi:10.1016/J.NEULET.2005.08.055

Parkening, T. A., Collins, T. J., Lau, I. F., and Saksena, S. K. (1982). The pituitary–ovarian complex in the aged anoestrous golden hamster. *J. Reprod. Fertil.* **64**, 37–46. doi:10.1530/JRF.0.0640037

Pozzan, T., Magalhaes, P., and Rizzuto, R. (2000). The comeback of mitochondria to calcium signalling. *Cell Calcium* **28**, 279–283. doi:10.1054/CECA.2000.0166

Quintero, M., Colombo, S. L., Godfrey, A., and Moncada, S. (2006). Mitochondria as signaling organelles in the vascular endothelium. *Proc. Natl Acad. Sci. USA* **103**, 5379–5384. doi:10.1073/PNAS.0601026103

Reynier, P., May-Panloup, P., Chretien, M. F., Morgan, C. J., Jean, M., Savagner, F., Barriere, P., and Malthiery, Y. (2001). Mitochondrial DNA content affects the fertilizability of human oocytes. *Mol. Hum. Reprod.* **7**, 425–429. doi:10.1093/MOLEHR/7.5.425

Rienzi, L., Ubaldi, F. M., Iacobelli, M., Minasi, M. G., Romano, S., Ferrero, S., Sapienza, F., Baroni, E., Litwicka, K., and Greco, E. (2008). Significance of metaphase II human oocyte morphology on ICSI outcome. *Fertil. Steril.* **90**, 1692–1700. doi:10.1016/J.FERTNSTERT.2007.09.024

Roy, M. K., Thalang, V. N., Trakoontivakorn, G., and Nakahara, K. (2004). Mechanism of mahanine-induced apoptosis in human leukemia cells (HL-60). *Biochem. Pharmacol.* **67**, 41–51. doi:10.1016/J.BCP.2003.07.021

Sathananthan, A. H. (1997). Ultrastructure of the human egg. *Hum. Cell* **10**, 21–38.

Simsek-Duran, F., Li, F., Ford, W., Swanson, R. J., Jones, H. W., Jr, and Castora, F. J. (2013). Age-associated metabolic and morphologic changes in mitochondria of individual mouse and hamster oocytes. *PLoS One* **8**, e64955. doi:10.1371/JOURNAL.PONE.0064955

Speakman, J. R., Talbot, D. A., Selman, C., Snart, S., McLaren, J. S., Redman, P., Krol, E., Jackson, D. M., Johnson, M. S., and Brand, M. D. (2004). Uncoupled and surviving: individual mice with high metabolism have greater mitochondrial uncoupling and live longer. *Aging Cell* **3**, 87–95. doi:10.1111/J.1474-9728.2004.00097.X

Tang, D. W., Fang, Y., Liu, Z. X., Wu, Y., Wang, X. L., Zhao, S., Han, G. C., and Zeng, S. M. (2013). The disturbances of endoplasmic reticulum calcium homeostasis caused by increased intracellular reactive oxygen species contributes to fragmentation in aged porcine oocytes. *Biol. Reprod.* **89**, 124. doi:10.1095/BIOLREPROD.113.111302

Tatone, C., Di Emidio, G., Barbaro, R., Vento, M., Ciriminna, R., and Artini, P. G. (2011). Effects of reproductive aging and postovulatory aging on the maintenance of biological competence after oocyte vitrification: insights from the mouse model. *Theriogenology* **76**, 864–873. doi:10.1016/J.THERIOGENOLOGY.2011.04.017

Thouas, G. A., Trounson, A. O., Wolvetang, E. J., and Jones, G. M. (2004). Mitochondrial dysfunction in mouse oocytes results in preimplantation embryo arrest *in vitro*. *Biol. Reprod.* **71**, 1936–1942. doi:10.1095/BIOLREPROD.104.033589

Van Blerkom, J. (2008). Mitochondria as regulatory forces in oocytes, preimplantation embryos and stem cells. *Reprod. Biomed. Online* **16**, 553–569. doi:10.1016/S1472-6483(10)60463-4

Van Blerkom, J. (2011). Mitochondrial function in the human oocyte and embryo and their role in developmental competence. *Mitochondrion* **11**, 797–813. doi:10.1016/J.MITO.2010.09.012

Van Blerkom, J., Davis, P., Mathwig, V., and Alexander, S. (2002). Domains of high-polarized and low-polarized mitochondria may occur in mouse and human oocytes and early embryos. *Hum. Reprod.* **17**, 393–406. doi:10.1093/HUMREP/17.2.393

Weakley, B. S. (1976). Variations in mitochondrial size and ultrastructure during germ cell development. *Cell Tissue Res.* **169**, 531–550. doi:10.1007/BF00218151

Weakley, B. S., and James, J. L. (1982). Differentiation of endoplasmic reticulum in the developing oocyte of the golden hamster (*Mesocricetus auratus*). *Cell Tissue Res.* **223**, 127–139. doi:10.1007/BF00221504

Weibel, E. R., Kistler, G. S., and Scherle, W. F. (1966). Practical stereological methods for morphometric cytology. *J. Cell Biol.* **30**, 23–38. doi:10.1083/JCB.30.1.23

Xia, P. (1997). Intracytoplasmic sperm injection: correlation of oocyte grade based on polar body, perivitelline space and cytoplasmic inclusions with fertilization rate and embryo quality. *Hum. Reprod.* **12**, 1750–1755. doi:10.1093/HUMREP/12.8.1750

Yamauchi, Y., Yanagimachi, R., and Horiuchi, T. (2002). Full-term development of golden hamster oocytes following intracytoplasmic sperm head injection. *Biol. Reprod.* **67**, 534–539. doi:10.1095/BIOLREPROD67.2.534

Zeng, H. T., Yeung, W. S., Cheung, M. P., Ho, P. C., Lee, C. K., Zhuang, G. L., Liang, X. Y., and O, W. S. (2009). *In vitro*-matured rat oocytes have low mitochondrial deoxyribonucleic acid and adenosine triphosphate contents and have abnormal mitochondrial redistribution. *Fertil. Steril.* **91**, 900–907. doi:10.1016/J.FERTNSTERT.2007.12.008