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Effects of ovarian hyperstimulation on mitochondria in oocytes and early embryos

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Abstract. A mouse model was used to compare the number and function of mitochondria in oocytes and embryos obtained by superovulation and in a natural cycle (control group). The superovulation group had a higher number of total oocytes, MII oocytes, embryos with two pronuclei, 2-cell embryos and blastocysts than the control group ($P < 0.05$ for all). The superovulation group had high proportion of MII oocytes with low number of mitochondrial (mt) DNA copies. The average number of mtDNA copies, ATP level and mitochondrial membrane potential ($\triangle\Psi$m) in MII oocytes in the superovulation were lower than in the control group ($P < 0.05$ for all). However, at the blastocyst stage, mean mtDNA copies, ATP level and $\triangle\Psi$m did not differ significantly between the two groups. These results suggest that ovarian hyperstimulation does not cause damage to the mitochondria in eggs but, rather, more eggs with poor mitochondrial quality are recruited, resulting in a decline in average mitochondrial quality.

Additional keywords: mitochondrial DNA, mitochondrial membrane potential.

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Introduction

For IVF, eggs are often harvested through superovulation via a high gonadotropin (Gn) dose. However, numerous studies have suggested that eggs obtained via superovulation have low developmental potential with abnormal spindle structures (Van Blerkom and Davis 2001) and an abnormal distribution of actin and cortical granules (Lee *et al.* 2005). The blastocyst production rate after superovulation is low, which is accompanied by a small inner cell mass (McKiernan and Bavister 1998; Edwards *et al.* 2005) and increased chance of polyploid embryos (Ma *et al.* 1997). Conversely, some believe that ovarian hyperstimulation itself is harmless to eggs and that only more low-quality eggs are recruited (Gosden and Lee 2010).

Ovarian hyperstimulation acts on antral follicle development, especially on the late stage, which is a critical period for cytoplasm maturation. Mitochondria are important cytoplasmic organelles involved in the regulation of intracellular metabolism and apoptosis. Mitochondrial function is essential for the normal activity of the oocyte cytoskeleton (Katayama *et al.* 2006) and normal chromosome segregation (Van Blerkom 2011). Both play an important role in fertilisation and embryonic development (Van Blerkom 2008). The zygotic genome is not activated, and no mitochondrial replication occurs, before embryo implantation (Dumollard *et al.* 2007). Therefore, the energy for early embryo development is totally dependent on the oocyte mitochondrial reserve.

During antral follicle development, oocyte mitochondria replicate considerably (Dumollard *et al.* 2007). It is unknown whether ovarian hyperstimulation during this sensitive period interferes with the natural changes occurring in the oocyte mitochondria, and whether ovarian hyperstimulation may result in a low development potential of oocytes and embryos due to mitochondrial replication injury.

In addition, the mitochondrion is the only organelle that contains extranuclear genetic material. Compared with nuclear DNA, mitochondrial (mt) DNA does not have any histone protection, repair or recombination mechanisms. Because of sperm mitochondrial ubiquitinoylation in ooplasma, zygotic mtDNA is only inherited from the mother (Sutovsky *et al.* 1999). Therefore, oocyte mitochondrial mutations are an

important issue for both embryo developmental potential and offspring health. During superovulation, changes in the ovarian metabolic environment are much more severe than during normal physiological follicular development. Jancar *et al.* (2008) reported that ovarian stimulation increases the incidence of apoptotic granulosa cells, but has no effect on the level of production of reactive oxygen species (ROS). Chao *et al.* (2005) reported that after repeated superovulation in mice, oxidative damage products and mtDNA mutations in ovarian tissue are increased. Gibson *et al.* (2005) reported that in rhesus monkeys mtDNA deletion is increased in eggs obtained through ovarian hyperstimulation. Because oxidative damage is a leading cause of mtDNA mutations, the question arises whether ovarian hyperstimulation may pose a potential risk to reproduction and the health of offspring due to ovarian oxidative damage and subsequent mutation of oocyte mtDNA.

In the present study, a mouse model was used to compare the degree of maturation, IVF rate and embryo development of oocytes obtained by superovulation and in a natural cycle. The number and function of mitochondria in oocytes and embryos at different development stages were compared between the two groups. The morphology, distribution and genetic heterogeneity of mitochondria in oocytes, and oxidative products of the ovarian tissue, were also compared between the two groups.

Materials and methods

This study was approved by the Institutional Review Board and the Institutional Animal Care and Use Committee (IACUC) of the School of Medicine, Zhejiang University, Hangzhou, China. Portions of the methods have been reported previously (Shu *et al.* 2013).

Study animals, observation of the oestrous cycle and ovarian stimulation

Female (8–10 weeks of age; weight 20–25 g) and male (12–13 weeks of age; weight 30–35 g) C57BL/6J mice purchased from the Experimental Animal Center of Zhejiang University were used in the present study. Mice were raised under a 14-h light–10-h dark cycle at 24°C and had free access to food and drinking water. Vaginal smears of female mice were obtained daily at noon. After haematoxylin and Alcian blue staining, slides were observed and mice were categorised as being in dioestrus, pro-oestrus, oestrus or metoestrus.

In the control group, with typical smear manifestations of the oestrous cycle at noon, mice were injected with human chorionic gonadotropin (hCG), 10 IU, i.p., per mouse on the same day at 1800 hours. In the superovulation group, mice in dioestrus were administered 10 IU, i.p., pregnant mare's serum gonadotropin (PMSG), followed by 10 IU hCG 48 h later. In both groups, eggs were obtained 14 h after hCG injection and the cumulus–oocyte complex (COC) was collected for IVF and embryo culture.

Oocyte retrieval, fertilisation and embryo culture

After an intraperitoneal injection of an overdose of 1% pentobarbital at 0.01 mL g^{-1} bodyweight, mice were killed by cervical dislocation. The fallopian tube was cut and placed in modified human tubal fluid (mHTF; Irvine Scientific, Santa Ana, CA, USA). The COCs were released by needle puncture to the oviduct magnum, then were transferred to HTF.

To collect oocytes, COCs were treated with 80 IU mL^{-1} hyaluronidase (Sigma-Aldrich, St Louis, MO, USA) for 2–3 min before COCs were transferred to mHTF using a fine glass needle. COCs were washed with mHTF to remove granulosa cells. Oocyte morphology was observed under a microscope. If polar body discharge was observed, the egg was classified as an MII oocyte.

For IVF, the COC was cultured in a droplet and covered by mineral oil. Spermatozoa harvested from the epididymis of a male mouse were collected in HTF medium containing 10% serum substitute supplement (SSS) and then cultured in an incubator with 5% CO_2 at 37°C for 1 h to allow capacitation. The sperm concentration was adjusted to 1×10^6 mL^{-1} and 6 μL sperm solution was added to each COC droplet.

Fertilisation status was observed 8 h after insemination. The oocyte was considered to be fertilised normally when two pronuclei (PN; a male PN and female PN) appeared. The zygote was then transferred to HTF medium containing 10% SSS and cultured continuously. Embryo development was observed once a day. The fertilisation rate (number of 2PN embryos/total number of oocytes obtained), cleavage rate (number of 2-cell embryos/number of 2PN embryos) and blastocyst formation rate (number of blastocysts/number of 2PN embryos) were calculated. All 2PN and 2-cell embryos and blastocysts were collected for examination of embryo mtDNA copy number, ATP detection and JC-1 staining.

Analysis of mtDNA copy number in eggs and embryos

In a mature oocyte, each mitochondrion contains one to two mtDNA copies. Thus, the number of mtDNA copies can be used as an index of the quantity of mitochondria (Shoubridge and Wai 2007). The methods used for the analysis of mtDNA copy number in eggs and embryos were as described previously (Shu *et al.* 2013). Briefly, purified and absolute quantified polymerase chain reaction (PCR) products of the mouse mtDNA 2567–2723 fragment (157 bp) were prepared using the following primers: CGAAAGGACAAGAGAAATAGAG (forward) and GAACAAGGTTTTAAGTCTTACGCA (reverse). The primers were designed according to the GenBank mtDNA standard sequence (GI: 33115104) for C57 mice and produced by Shanghai Sangon Biological Engineering Technology Services (Shanghai, China). Serial external standard templates with a known copy number were produced by serial dilutions of the purified product.

Pooled oocytes or embryos were lysed by proteinase K-containing buffer in a PCR tube, and the average number of mtDNA copies was determined by real-time PCR simultaneously with the serial external standard calibration products. The reaction was performed with 5 μL SYBR, 0.1 μL forward primer (10 μM), 0.2 μL reverse primer (10 μM), 3 μL mtDNA template in DNA lysis buffer and 1.6 μL distilled water. The reaction conditions were 94°C denaturation for 5 min, followed by 40 cycles of 94°C denaturation for 30 s, 61°C annealing for 30 s and 72°C extension for 30 s.

ATP detection

The ATP detection methods used in the present study were as described previously (Shu *et al.* 2013). ATP assays were performed with an ATP Determination Kit (A22066; Molecular Probes, Life Technologies, Carlsbad, CA, USA). Changes in chemiluminescence were measured with a Thermo Scientific Luminoskan Ascent chemiluminescence plate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Observation of mitochondrial membrane potential

The methods used for the determination of mitochondrial membrane potential ΔΨm were as described previously (Shu *et al.* 2013). The fluorescent dye JC-1 (catalogue number 1130–5; Biovision) fluoresces red for a high ΔΨm, whereas if ΔΨm is low the dye fluoresces green. Red and green fluorescence distribution and intensity were evaluated using a two-photon confocal microscope (BX61W1-FV1000; Olympus). Each egg was measured at an equatorial axis section. The light excitation and emission wavelengths for red and green fluorescence were 559 and 572 nm, respectively, and 488 and 520 nm, respectively. ImageJ software was used to calculate the ratio of red to green fluorescence intensity (ΔΨm).

Observation of mitochondria distribution in oocytes

MitoTracker Red FM (catalogue no. M2242; Invitrogen) was used for observation of mitochondria distribution. MitoTracker Red is a fluorescent dye that passes through the cell membrane and accumulates in mitochondria. It is oxidised by active mitochondria and exhibits red fluorescence. Mitochondrial staining with MitoTracker does not depend on ΔΨm and, unlike JC-1 staining, can be used to quantify the number of mitochondria.

Oocytes or embryos were transferred into HTF containing 300 nM MitoTracker Red and incubated in the dark at 37°C for 60 min. They were then rinsed three times with mHTF before being placed in a Petri dish for immediate observation. A laser confocal microscope was used to observe the excitation and emission of MitoTracker Red FM probes at 543 and 633 nm, respectively. Each oocyte was scanned at an equatorial plane with a slice thickness of 2 μm. The distribution of mitochondria in oocytes was categorised as follows: (1) uniform distribution, whereby the mitochondria were distributed throughout the cytoplasm as if they were fine particles; (2) cluster-like distribution, whereby large particles were present and the mitochondria were distributed unevenly in the ooplasm or under the plasma membrane; and (3) polar aggregation, whereby large mitochondrial particles gathered in one hemisphere and small mitochondrial particles were found in the other hemisphere.

Observation of the mitochondrial ultrastructure of oocytes

A total of 12 MII oocytes was randomly selected from each of the control and superovulation groups for observation of mitochondrial ultrastructure by electron microscopy (Shu *et al.* 2013). After fixation, staining and dehydration, the specimens were soaked in 1 : 1 acetone : Epon812 for 1.5 h, embedded and placed into a polymerisation reactor (37°C, 24 h; 45°C, 24 h; 60°C, 48 h). Ultrathin sections (120 nm) were cut and stained with 4% uranyl acetate for 20 min and lead citrate for 5 min. The samples were then observed and photographed under a TECNAI–10 Phillips electron microscope (80–100 kV).

Detection of mtDNA D-loop region heterogeneity

Mouse mtDNA has a total of 16 299 bp and the D-loop region fragments were selected for heterogeneity testing. The D-loop region is located at nucleotides 15 423–16 299. It is a mutation hot spot and is the regulatory region for mitochondrial replication and transcription. Single oocytes were transferred into a sterile PCR tube and oocyte lysis and inactivation of proteinase K procedures were performed similar to those described above for analysis of mtDNA copy number. Samples were stored at −80°C until use.

Denaturing HPLC (DHPLC) was used for heterogeneity detection. DNA double strands dissociate when heated to 95°C. During a slow cooling process, samples containing heterogeneous DNA produce hybridisation products. The column hold-up time of the heteroduplex with a mutant sequence is shorter than that of the corresponding homoduplex. Therefore, samples with the mutant sequence exhibit two peaks (heteroduplex and homoduplex) and samples without the mutant sequence only produce the homoduplex peak. Under ideal separation conditions, fragments containing a 1% difference in size can be separated. The temperatures for denaturation in the present study were: A, fragment 15 308–15 703, denaturation at 53.8°C; B, fragment 15 632–15 977, denaturation at 57.8°C; and C, fragment 15 961–16 269, denaturation at 54.2°C. Five mice from each group were used in the experiment and all oocytes harvested from mice in the control and superovulation groups were tallied; there were 41 and 103 eggs, respectively.

Detection of oxidative products and antioxidant capacity of ovarian tissue

The antioxidant capacity of ovarian tissue has been evaluated previously (Kankofer *et al.* 2013). Briefly, 8-hydroxydeoxyguanosine (8-OH-dG) is the product of oxidative damage to DNA caused by free radicals. Advanced oxidation protein products (AOPP) are produced by oxidative damage to proteins caused by free radicals. Maleic dialdehyde (MDA) is a lipid peroxidation product. Total antioxidant capacity (T-AOC) is a measure of the antioxidant capacity of two systems: enzymatic and non-enzymatic.

Both ovaries of mice in the two groups (10 mice from each group) were removed before the mice were injected with hCG and were rapidly frozen in liquid nitrogen for storage. The ovaries were taken out of the liquid nitrogen and placed on ice before the experiment. After addition of 400 μL phosphate-buffered saline (PBS), the ovaries were ground in a grinder and centrifuged at 667*g* for 10 min at 4°C. The supernatant was collected for measurements. 8-OH-dG was detected using ELISA (8-OH-dG detection kit; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. AOPP was also detected using ELISA (AOPP detection kit; R&D Systems). MDA was detected using a thiobarbituric acid (TBA) assay (MDA detection kit; Nanjing Jiancheng Bioengineering Institute). MDA can condense with TBA to form red products. The product has a maximum absorption at 532 nm. The test was performed and optical density (OD) was measured

Table 1. Comparison of oocyte recovery and embryo development in the superovulation and control groups

Non-normally distributed data are presented as median values with the interquartile range in parentheses. The significance of differences between groups was determined using the Mann–Whitney *U*-test. 2PN, two pronuclei

	Superovulation group ($n = 22$ mice)	Control group ($n = 22$ mice)	*P*-value
Harvested oocytes and embryos per mouse			
Oocytes retrieved	27 (21.00–34.00)	9 (7.00–10.00)	<0.001
MII oocytes	23 (18.00–28.30)	8 (7.00–9.00)	<0.001
2PN embryos	18.5 (11.00–23.50)	7 (5.50–8.00)	<0.001
2-cell embryos	14.5 (7.80–17.30)	5 (4.50–6.00)	<0.001
Blastocysts	9.5 (6.00–12.30)	4 (3.00–4.50)	<0.001
Egg maturation rate (no. MII oocytes/oocytes retrieved)	83.8% (79.4%–87.7%)	91.67% (88.9%–100%)	<0.001
Fertilisation rate (no. 2PN embryos/oocytes retrieved)	66.3% (64.3%–67.9%)	80% (73.2%–86.6%)	<0.001
Cleavage rate (2-cell embryos/2PN embryos)	74.5% (72.2%–79.5%)	80% (71.4%–83.3%)	0.085
Blastocyst rate (no. blastocysts/2PN embryos)	50% (50%–54.9%)	60% (50%–66.7%)	0.011

Table 2. Comparison of the mitochondrial (mt) DNA copies, ATP levels and mitochondrial membrane potential ($\Delta\Psi$m) in the superovulation and control groups

Normally distributed data are presented as the mean ± s.d.; non-normally distributed data are given as median values with the interquartile range in parentheses. The significance of differences between groups was determined using the two-sample *t*-test for normally distributed data and the Mann–Whitney *U*-test for non-normally distributed data. 2PN, two pronuclei

	Superovulation group	Control group	*P*-value
mtDNA copies ($\times 10^5$ per oocyte or embryo)	$n = 10$ mice	$n = 10$ mice	
MII oocytes	1.19 (0.56–2.06)	2.76 (1.44–4.53)	<0.001
2PN embryos	1.40 (0.95–2.76)	2.04 (1.33–4.54)	0.021
2-cell embryos	2.02 (1.42–3.04)	2.44 (1.60–3.57)	0.285
Blastocysts	2.25 (1.26–4.24)	3.84 (1.30–7.86)	0.073
ATP levels (per oocyte or embryo)	$n = 6$ mice	$n = 6$ mice	
MII oocytes	33.65 ± 20.65	45.41 ± 20.16	0.001
2PN embryos	69.98 ± 44.47	82.16 ± 46.15	0.181
2-cell embryos	83.56 ± 26.22	86.43 ± 25.97	0.631
Blastocyst	170.60 ± 37.52	179.60 ± 49.54	0.738
$\Delta\Psi$m	$n = 6$ mice	$n = 6$ mice	
MII oocytes	1.13 ± 0.32	1.39 ± 0.24	<0.001
2-cell embryos	1.33 (1.17–1.55)	1.44 (1.35–1.53)	0.015
Blastocyst	1.53 ± 0.32	1.58 ± 0.24	0.513

according to the manufacturer's instructions. T-AOC was detected using a colorimetric method. Antioxidants were used to reduce Fe^{3+} to Fe^{2+}; the latter can form a stable complex with a phenanthroline. The whole T-AOC detection procedure was performed according to the T-AOC measurement kit instructions (Nanjing Jiancheng Bioengineering Institute) and OD was measured at 520 nm to determine the concentration of T-AOC.

Statistical analysis

Categorical data are presented as numbers with percentages in parentheses, whereas continuous data are presented as the mean ± s.d. Differences between groups were analysed by Fisher's exact test for categorical data, and by the two-sample *t*-test for continuous data. Continuous data with a non-normal distribution are presented as median values with the interquartile range in parentheses and were analysed by the Mann–Whitney *U*-test. All statistical assessments were two-tailed and $P < 0.05$ was considered significant. Statistical analyses were performed using SPSS 18.0 (SPSS Inc., Chicago, IL, USA).

Results

In the control group, we attempted to harvest oocytes from 118 mice in which the oestrus cycle was observed; oocytes were obtained from 90 mice, giving a success rate of egg retrieval of 76.3%. In the superovulation group, we attempted to harvest oocytes from 95 mice; oocytes were obtained from 90 mice, giving a success rate of egg retrieval of 94.7%, which was significantly higher than that of the control group ($P < 0.01$). The oocytes from all mice were randomly used in subsequent experiments. In all experiments, all oocytes collected were used; no oocytes were discarded.

A comparison of oocyte recovery and embryo development between the superovulation and control groups (22 mice in each group) is given in Table 1. The superovulation group had a higher number of oocytes retrieved, MII oocytes, 2PN embryos, 2-cell embryos and blastocysts per mouse than the control group ($P < 0.05$ for all). However, the egg maturation rate, fertilisation rate and blastocyst rate were all lower in the superovulation group ($P < 0.05$ for all; Table 1). Table 2 shows that the median

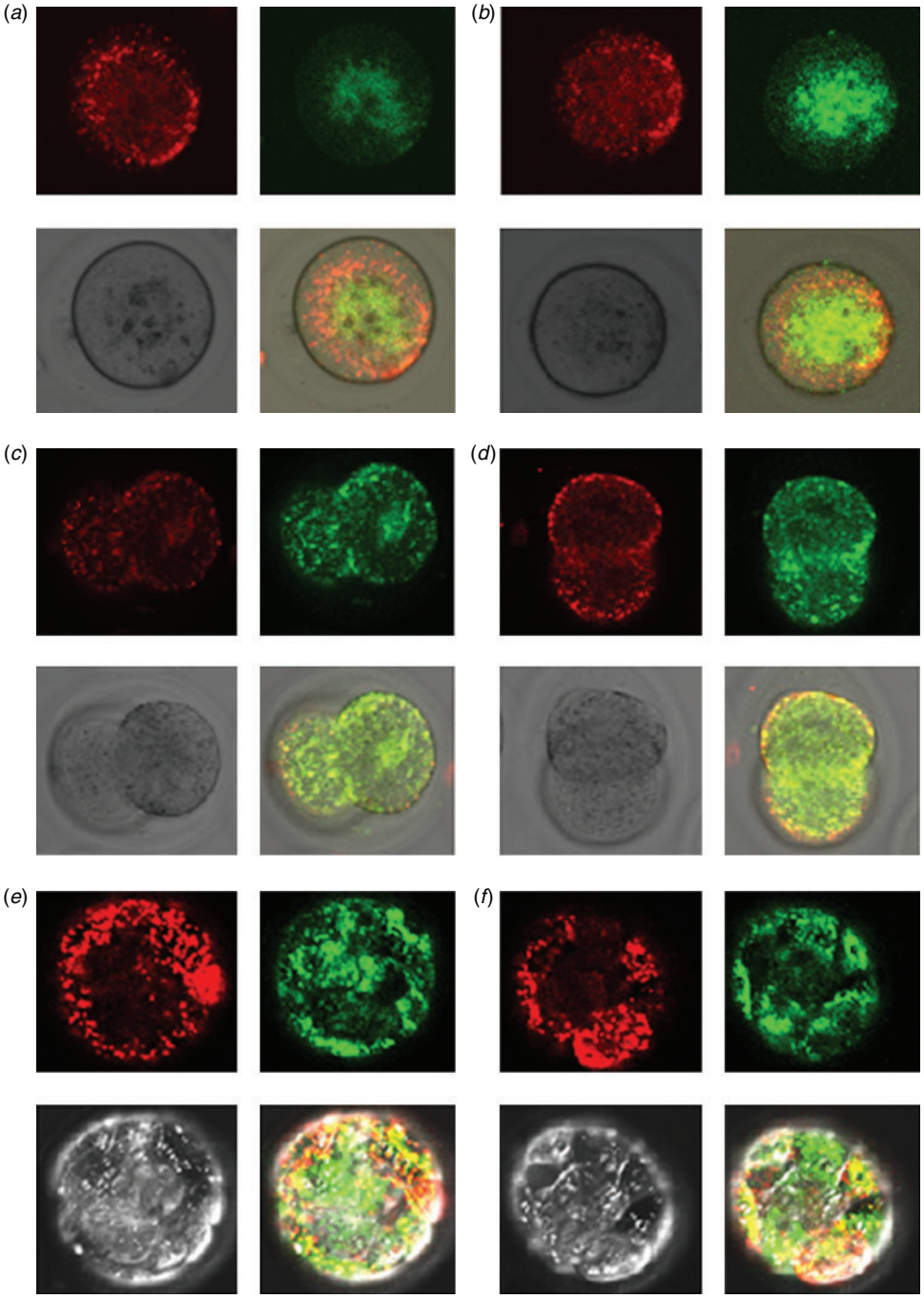

Fig. 1. JC-1 staining pattern of eggs and embryos. (*a*) Control group MII eggs; (*b*) superovulation (SOV) group MII eggs; (*c*) control group 2-cell embryos; (*d*) SOV group 2-cell embryos; (*e*) control group blastocysts; (*f*) SOV group blastocysts. JC-1 red fluorescence shows that hyperpolarised mitochondria are mainly distributed under the cell membrane or under the trophoblast layer. In each panel, the four images show red fluorescence, green fluorescence, brightfield and merged (red + green) images.

number of mtDNA copies of MII oocytes and 2PN embryos in the superovulation group was lower than in the control group ($P < 0.05$ for both). The ATP levels of MII oocytes in the superovulation group were lower than in the control group (33.65 ± 20.65 vs 45.41 ± 20.16 fmol, respectively; $P = 0.001$). The $\Delta\Psi$m of MII oocytes and 2PN embryos of the superovulation group was lower than in the control group (1.13 ± 0.32 vs 1.39 ± 0.24 for MII oocytes, respectively ($P < 0.001$); 1.33

Table 3. Proportions of MII oocytes with different mitochondrial (mt) DNA content

Data are presented as number (percentage) by group. The significance of differences between groups was determined using Fishers' exact test.

	Superovulation group ($n = 10$ mice)	Control group ($n = 10$ mice)	*P*-value
Total no. MII oocytes	270	76	
mtDNA copy number			<0.001
$<1 \times 10^5$	112 (41.5%)	10 (13.2%)	<0.001
1×10^5 to 5×10^5	139 (51.5%)	50 (65.8%)	0.027
5×10^5 to 10×10^5	15 (5.6%)	13 (17.1%)	0.001
$>10 \times 10^5$	4 (1.5%)	3 (3.9%)	0.182

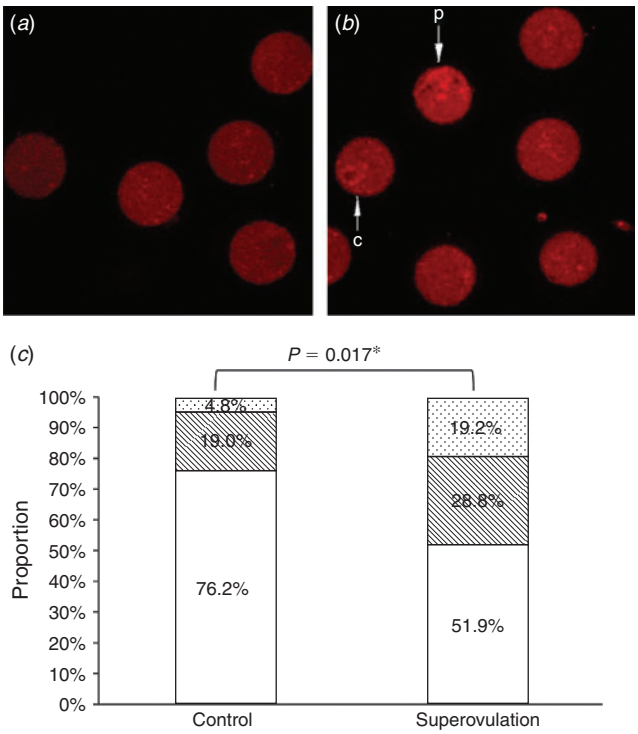

Fig. 2. Mitochondrial distribution in MII oocytes by MitoTracker Red FM staining. (*a*) Control group MII oocytes were uniform in size with uniform distribution of mitochondrial particles. (*b*) Superovulation group MII oocytes exhibited mitochondrial polarity: cluster-like distribution (c) and polar aggregation (p). (*c*) Mitochondrial distribution patterns between the superovulation and control groups showing the proportion of oocytes with mitochondria exhibiting uniform distribution (□) and cluster-like (▨) and polar aggregations (⊡). Data are presented as the mean ± s.e.m. Dispersion differed significantly between the two groups ($P < 0.05$).

(1.17–1.55) vs 1.44 (1.35–1.53) for 2PN embryos, respectively ($P = 0.015$)). At the blastocyst stage, mtDNA copies, ATP level and ΔΨm were not significantly different between the two groups (Table 2). The JC-1 staining pattern of eggs and embryos in the two groups is shown in Fig. 1. JC-1 red fluorescence showed that hyperpolarised mitochondria were mainly distributed under the cell membrane or under the trophoblast layer.

The proportion of MII oocytes with a different mtDNA content level was significantly different between the superovulation and control groups ($P < 0.001$; Table 3). Approximately 41.5% of MII oocytes in the superovulation group had a small number of mtDNA copies ($<1 \times 10^5$), compared with only 13.2% of MII oocytes in the control group.

The distribution pattern of mitochondria in MII oocytes was also different between the two groups. In the control group, 76.2% of MII oocytes had a uniform distribution of mitochondrial particles, whereas in the superovulation group 51.9% of MII oocytes exhibited a uniform mitochondrial distribution and 48.1% exhibited distribution polarity (Fig. 2; $P = 0.017$).

Observation of mitochondrial ultrastructure showed that all selected MII oocytes in the control group (12/12) had abundant mitochondria in a spherical shape, and the mitochondria had a high matrix density and a small number of ridges (Fig. 3). The endoplasmic reticulum vesicles were surrounded by many crescent-like mitochondria. In the superovulation group, the mitochondrial ultrastructure in 83.3% of selected oocytes (10/12) was similar to that in the control group; however, in 16.7% of oocytes (2/12) the mitochondria were sparse and small in size, and they lacked a condensed mitochondrial structure around the endoplasmic reticulum vesicles.

Heterogeneity analysis revealed no heterogeneity in the three D-loop region fragments (15 308–15 703, 15 632–15 977 and 15 961–16 269) in oocytes in either group.

Results of the analysis of oxidative damage and the antioxidant capacity of ovarian tissue in the two groups are given in Table 4. T-AOC was significantly higher in the superovulation group than in the control group (16.59 ± 8.76 vs 9.55 ± 5.64 units mg^{-1} protein, respectively; $P = 0.047$), whereas there were no significant differences in AOPP, 8-OH-dG and MDA between the two groups.

Discussion

The results of the present study using a murine model showed that oocytes produced by superovulation are more likely to be immature and have a low number of mitochondria with decreased function, which are likely to be selected out during the fertilisation and cleavage process. However, there was no significant difference between the groups in the number and function of blastocyst mitochondria, indicating that high-quality oocytes can be obtained through ovarian hyperstimulation. The results support the hypothesis that the low embryo development rate of oocytes obtained after high-dose Gn is due to recruitment of a large number of low-potential eggs rather than egg damage induced by high-dose Gn.

Ge *et al.* (2013) found that superovulation results in a lower mean number of mitochondria and inferred that ovarian hyperstimulation can destroy mitochondria replication. However, in the present study these results are primarily attributed to the higher percentage of eggs with lower mtDNA content in the superovulation group. Mitochondria do not replicate before embryo implantation; thus, the number of mitochondria in a single early embryo can reflect the number of mitochondria in the oocyte, its developmental origin (Thundathil *et al.* 2005; Wai *et al.* 2010). In the present study, the mtDNA content

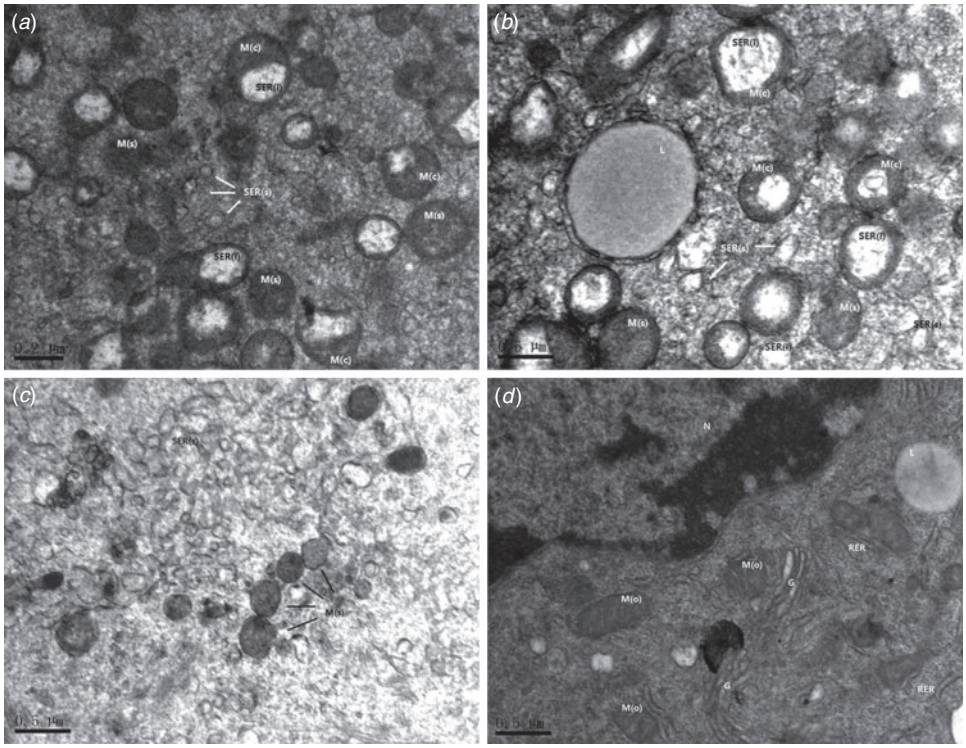

Fig. 3. Mitochondrial ultrastructure in oocytes as determined by electron microscopy. (*a*) Eggs in the control group are rich in mitochondria; the mitochondria are spherical and have a high matrix density and few ridges; the endoplasmic reticulum vesicles are surrounded by some crescent-like mitochondria. (*b*) Ten eggs in the superovulation group were rich in mitochondria and had similar structures to those seen in the control group (*a*). (*c*) Two eggs in the superovulation group had few, small mitochondria, and there were no mitochondria–endoplasmic reticulum complexes in the visual field. (*d*) Follicular cells showed abundant mitochondria and Golgi apparatus, a nucleus in a granulosa cell was observed and the mitochondria were oval and had a low matrix density and rich transverse ridges. M(s), spherical mitochondria; M(c), crescent-like mitochondria; M(o), oval mitochondria with rich ridges; SER(s), smooth endoplasmic reticulum small vesicle; SER(l), SER large vesicle; RER, rough endoplasmic reticulum; L, lipid droplets; G, Golgi apparatus; N, nucleus.

Table 4. Comparison of oxidative damage and antioxidant capacity of ovarian tissue

Data are presented as number (percentage), mean ± s.d. or as median values with the interquartile range in parentheses. AOPP, advanced oxidation protein products; 8-OH-dG, 8-hydroxy-deoxyguanosine; MDA, maleic dialdehyde; T-AOC, total antioxidant capacity

	Superovulation group ($n = 10$ mice)	Control group ($n = 10$ mice)	*P*-value
AOPP (mmol mg^{-1} protein)	44.91 (41.39–50.35)	40.95 (39.79–46.64)	0.123
8-OH-dG (pg mg^{-1} protein)	1304.73 ± 309.87	1109.43 ± 86.90	0.177
MDA (nmol mg^{-1} protein)	3.73 ± 1.31	3.83 ± 1.37	0.868
T-AOC (units mg^{-1} protein)	16.59 ± 8.76	9.55 ± 5.64	0.047

increased gradually in the superovulation group from the MII oocyte to the 2PN, 2-cell and the blastocyst (Table 1), which verifies that a low number of mitochondrial copies has a negative impact on the fertilisation of oocytes and early embryo development. Santos *et al.* (2006) observed that the average number of mtDNA copies was 250 454 and 163 698 in human zygotes and non-fertilised eggs, respectively, compared with 44 629 in degraded egg. Zeng *et al.* (2007) compared the mtDNA copy numbers in oocytes with and without meiotic spindle formation and found that the number was lower in the latter and that the latter had a low fertilisation rate after intracytoplasmic sperm injection (ICSI).

Fertilisation and cleavage are energy-consuming processes that require energy support from mitochondrial ATP (Dumollard *et al.* 2004). Eggs with low mitochondrial function cannot mature and the tendency for an increase in ATP and $\Delta\Psi m$ from the MII oocyte to blastocyst stage confirm this. ATP levels and $\Delta\Psi m$ were significantly different at the MII oocyte stage

between the two groups, but no differences were observed at the blastocyst stage. Among the large number of oocytes recruited after Gn administration, the percentage of oocytes with a low mitochondrial copy number was high, and we speculate that the oocytes were eliminated because the ATP generated by the mitochondria did not meet the requirements for fertilisation and cleavage. In addition to an insufficient number of mitochondria, the low $\Delta\Psi$m of the oocytes in the superovulation group may be a possible reason for the low ATP synthesis of oocytes. $\Delta\Psi$m is low when mitochondrial metabolism is low and is high when the metabolic rate is high. The decline in the average $\Delta\Psi$m of oocytes in the superovulation group is possibly due to low mitochondrial function in some oocytes obtained through superovulation, or mitochondrial membrane injury in low-potential oocytes and subsequent proton leakage.

Mitochondria distribution analysis with MitoTracker Red and observation of mitochondrial ultrastructure with electron microscopy revealed that the superovulation group had more variation in oocyte mitochondrial quality, a greater proportion of oocytes with cluster or polar aggregation and therefore a greater chance of collecting eggs with poor ooplasm quality with few, small mitochondria or a lack of mitochondrion–endoplasmic reticulum complexes.

The D-loop region regulates mtDNA replication and transcription (Fish *et al.* 2004) and mutations in this region are related to infertility and abortion (Parsons *et al.* 1997; Seyedhassani *et al.* 2010). D-Loop heterogeneity has been positively associated with age (Sondheimer *et al.* 2011), suggesting that long-term repeated oxidative damage may produce mtDNA mutations. No heterogeneity in the D-loop region of the mtDNA of oocytes was noted in the two groups, suggesting that the likelihood of superovulation causing mtDNA damage to the oocytes discharged in the current cycle is not high. Furthermore, we did not find increases in 8-OH-dG, AOPP or MDA in ovarian tissue after a single ovarian hyperstimulation. In contrast, the antioxidant capacity of hyperstimulated ovarian tissue was higher than that in the control group. Miyamoto *et al.* (2010) thought ovulation itself was an oxidative stress response and that superoxides contributed to the process of ovulation, and reported that after multiple ovulation 8-OH-dG content increased in oocytes, the mtDNA content of the eggs was decreased and mitochondria; distribution was abnormal.

The present study does have some limitations. An animal model was used, and so the results may not be applicable to human reproduction. Detection of heterogeneity may be affected by DHPLC sensitivity because the minimum heterogeneity that can be detected is only 1%. In addition, the fragments that can be used for detection are limited, and we did not examine the entire mtDNA genome. Previous studies (Ge *et al.* 2013) have performed comparisons using the same number of oocytes (i. e. comparing oocytes with excellent qualities obtained during a natural cycle with oocytes with different qualities harvested during ovarian hyperstimulation) and reported poorer results in the ovarian hyperstimulation group. We tried to compare the actual outcome of development between all the oocytes produced by a single individual during ovarian hyperstimulation and those produced by a single individual during a natural cycle. For this reason we choose the same number of mice for each group to test the efficiency of superovulation.

Conclusions

Superovulation increases the number of oocytes, but they are more likely to be immature and have a low number of mitochondria with decreased function and are likely to be selected out during the fertilisation and cleavage process. The number and function of blastocyst mitochondria did not differ between those obtained through ovarian hyperstimulation and a normal cycle, and a single ovarian hyperstimulation does not increase ovarian oxidative products and the mutation frequency of the mtDNA D-loop region. These results suggest that single ovarian hyperstimulation does not cause damage to the mitochondria in eggs, but rather more eggs of poor mitochondrial quality are recruited, resulting in a decline in average mitochondrial quality.

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