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Age-related changes in the mitochondria of human mural granulosa cells

Yifan Liu[1,†], Ming Han[2,3,†], Xiaoshuang Li[4,5], Hui Wang[1], Minyue Ma[1], Shihui Zhang[1], Yifan Guo[1], Shuling Wang[1], Yuanfen Wang[1], Na Duan[6], Bing Xu[1], Jingwen Yin[6], and Yuanqing Yao[1,*]

[1]Department of Obstetrics and Gynecology, Chinese PLA General Hospital and Chinese PLA Medical School, 28 Fuxing Road, Beijing 100853, China [2]Peking University Ditan Teaching Hospital, 8 Jingshun East Street, Beijing 100015, China [3]Institute of Infectious Diseases, Beijing Ditan Hospital, Capital Medical University, 8 Jingshun East Street, Beijing 100015, China [4]Naval Clinical College, Anhui Medical University, Meishan Road, Hefei 230032, China [5]Department of Obstetrics and Gynecology, PLA Navy General Hospital, 6 Fucheng Road, Beijing 100048, China [6]Clinical Medical College, Nankai University, 94 Weijin Road, Tianjin 300071, China

*Correspondence address. E-mail: yqyao@126.com.

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STUDY QUESTION: What changes in the mitochondria of human mural granulosa cells (mGCs) with maternal aging?

SUMMARY ANSWER: The mitochondrial membrane potential (MMP) and the ability of oxidative phosphorylation (OXPHOS) of mGCs declines with reproductive aging, accompanied with more abnormal mitochondria.

WHAT IS KNOWN ALREADY: Mitochondria play an important role in the dialogue between the mGCs and oocytes. However, the underlying mechanism of mitochondrial dysfunction in mGCs in aging is still poorly understood.

STUDY DESIGN SIZE, DURATION: In total, 149 infertile women underwent IVF in the ART Centre of the Chinese PLA General Hospital, China from September 2016 to May 2017. Two age groups were investigated: the young group (<38 years old) and the old group (≥38 years old).

PARTICIPANTS/MATERIALS, SETTING, METHODS: The mitochondrial ultrastructure of mGCs was observed by transmission electron microscopy, and real-time quantitative polymerase chain reaction was applied to quantify the mitochondrial DNA (mtDNA) copy number, 4977-bp deleted DNA and mRNA expression of mitochondrial ATP synthases ATP5A1 and ATP5I. MMP was detected by flow cytometry and fluorescence microscopy, respectively. Reactive oxygen species (ROS) was tested by flow cytometry. A luminometer was used to measure the ATP levels and western blot to analyse the OXPHOS complex.

MAIN RESULTS AND THE ROLE OF CHANCE: In the young group, mitochondria were mostly round or oval, with a few intact parallel tubular–vesicular cristae and homogenous matrix density, while elongated mitochondria were mainly observed in the old group, which had numerous cristae and more high-density matrix particles. Abnormal mitochondria were more common in aging women ($P = 0.012$). mtDNA relative copy number was positively correlated with maternal age ($r = 0.294$, $P = 0.009$) and we found no one with 4977-bp deleted mitochondria. JC-1 (dye used as an indicator of MMP) ratio in the old group was significantly lower than the young group (3.01 ± 0.21 vs 3.85 ± 0.27, $P = 0.033$). Intracellular ROS levels between the groups did not differ significantly ($P = 0.191$). The intracellular ATP level in the young group was 1.75-fold higher than that of the advanced-age group (7.17 ± 1.16 vs 4.15 ± 0.60, $P = 0.025$). The protein expression of ATP5A1, as one of five proteins of OXPHOS, decreased with aging ($P < 0.001$). ATP5A1 mRNA expression was negatively correlated with aging ($r = -0.341$, $P = 0.012$).

LIMITATIONS REASONS FOR CAUTION: The quantity of mGCs from some individual patient, especially an advanced-age individual, was small, which cannot meet the demands of all the detections.

[†]The authors consider that the first two authors should be regarded as joint first authors.

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WIDER IMPLICATIONS OF THE FINDINGS: mGCs dysfunction with aging is mainly linked to impaired mitochondrial function, especially OXPHOS function. Improving the OXPHOS ability in mGCs should be the focus in resolving infertility among advanced age women and making mGCs the proper mitochondria donor cells in the autologous mitochondria transplantation to oocytes.

STUDY FUNDING/COMPETING INTEREST(S): This work was supported by the grants of the National High Technology Research and Development Program of China, 863 Program No. SS2015AA020402, and the Key Projects of Military Medical Research, No. BWS11J058. There were no competing interests.

Key words: mural granulosa cells / mitochondria / maternal age / oxidative phosphorylation / adenosine triphosphate

Introduction

Female infertility and the risk of spontaneous miscarriage increase dramatically with maternal age, which has been the focus of ART research for decades. However, there is still no effective therapy to improve fertility outcomes.

A follicle consists of an oocyte and granulosa cells (GCs), including mural GCs (mGCs) lining the wall of the follicle and the cumulus cells (CCs) surrounding the oocyte. Oocytes regulate the GCs' functions by secreting factors, and GCs form the follicular microenviroment for oocyte developmental competence (Buccione et al., 1990; Gilchrist et al., 2008). Recently, the research scope has been extended to the age-related changes in the whole ovarian microenvironment. Some age-related changes in GCs have potential effects on oocyte quality (Ford, 2013; Tatone and Amicarelli, 2013). Mitochondria in GCs are critical to oocyte development and competence, and metabolize glucose into pyruvate which is then transported into the oocyte to produce ATP (Sutton-McDowall et al., 2010). Mitochondrial fraction decrease and increase in mitochondrial matrix density can be observed in the older group, 38–45 years (de Bruin et al., 2004). Reactive oxygen species (ROS) production is enhanced when the mitochondrial respiratory function declines with aging in GCs (May-Panloup et al., 2016; Tatone and Amicarelli, 2013). Coenzyme Q-dependent mitochondrial respiratory chain activity in mGCs is reduced with maternal age (Ben-Meir et al., 2015). The fraction of 4977-bp deletion in mitochondrial DNA (mtDNA) from mGCs over 38 years old increases significantly (Seifer et al., 2002). Thus, the mGCs from the ART patients provide a valuable model for investigating ovarian dysfunction due to aging.

The mGC microenvironment is similar to that of oocytes and is more conducive to analysis than CCs. Therefore, we investigate age-related changes in mitochondria in different aspects of mGCs. Based on our results, the decline in the mitochondrial oxidative phosphorylation (OXPHOS) function of mGCs due to aging is more significant than their copy number and genetic integrity, which elucidates ovary aging and reveals strategies to improve ART outcomes for advanced-age women.

Materials and Methods

Patients

mGC samples were obtained from 149 infertile women undergoing IVF in the ART Centre of the Chinese PLA General Hospital, China, from September 2016 to June 2017. Overall, 58 (38.9%) women with male origin, 74 (49.7%) infertile women with fallopian tube obstruction and 17 (11.4%) women with both male and tubal factors were recruited. Infertile women with polycystic ovarian syndrome, endometriosis, premature ovarian failure, chromosomal abnormalities or any chronic diseases of other systems were excluded from this study. Two age groups were investigated: the young group (<38 years old) and the old group (≥38 years old). Controlled ovarian hyperstimulation protocols are listed in Supplemental Table S1.

Ethical approval

This study was approved by the Institutional Review Board.

Isolation of GCS from follicle fluid

For each patient, mGCs were obtained from the pooled follicle fluid and isolated by density centrifugation. Follicle fluid was centrifuged at 400 *g* for 5 min, and sediments were resuspended in 5 mL of phosphate buffer solution. Cell suspensions were slowly layered on 7 mL Ficoll-Paque (Haoyang, China) and centrifuged at 590 *g* for 22 min. The mGC layer was washed three times with phosphate buffer solution (PBS, HyClone, USA). If red blood cells were not clearly separated from mGCs, red blood cell lysis buffer was added for 2 min at 4°C, and centrifuged at 400 *g* for 5 min. The mGCs were washed three times with PBS. Leucocyte contamination was evaluated by the percentage of CD45+ cells (CD45 antibody, BioLegend, USA) by flow cytometry (Becton Dickinson, USA), which was <6.73%. Characteristics of the isolated mGCs are listed in Supplemental Figures S1 and S2.

Transmission electron microscopy

The mGC samples were fixed with glutaraldehyde (3% in 0.1 mol/L cacodylate buffer, pH 7.4)(Fuchen, China) for 2 h and post-fixed in 1% osimium tetroxide (Ted Pella, USA) for 1 h before being dehydrated in acetone and embedded in SPI-Pon-812 (SPI Supplies, USA). After slicing, the 0.1-mm-thin sections were stained with uranyl acetate and lead citrate, and then observed with a JEM-1400 electron microscope (JEOL, Japan).

Quantification of mtDNA copy number and 4977-bp deleted mtDNA

Total DNA was extracted using a DNA extract kit (Omega, USA) according to the manufacturer's instruction. The mtDNA copy number in mGCs was determined by real-time quantitative polymerase chain reaction (qPCR) using a SYBR green assay on an 7500 Real-Time PCR system (Applied Biosystems, USA). The primer sequences and reaction conditions are listed in Supplemental Table SII. The relative mtDNA copy number was calculated by normalizing for the number of copies of the control gene using the comparative Ct method.

Detection of mitochondrial membrane potential

A fluorescent dye 5,5′,6,6′-tetrachloro-1,1′3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1, Beyotime, China), was used to measure mitochondrial membrane potential (MMP) of mGCs. mGCs were cultured with

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5 μg/mL JC-1 staining solution for 20 min at 37°C, avoiding direct light, and then washed twice with JC-1 staining buffer. mGCs in the positive group were incubated with 10 μM of carbonyl cyanide 3-chlorophenylhydrazone (CCCP) for 20 min and then stained with JC-1. The fluorescence intensity of mitochondrial JC-1 monomers (λEx 490 nm, λEm 530 nm) and aggregates (λEx 525 nm, λEm 590 nm) were detected immediately with a flow cytometry (Becton Dickinson, USA) and fluorescence microscopy (Olympus, Japan), respectively. The mean fluorescence intensity ratio of red to green was calculated.

Detection of intracellular ROS levels

A dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescent probe (Beyotime, China), was applied to detect the intracellular ROS levels. mGCs were resuspended using 10 μmol/L DCFH-DA and incubated for 20 min away from light at 37°C, and then washed three times with Dulbecco's Modified Eagle's Medium(DMEM)/F12 (HyClone, USA). Cell fluorescence intensity was detected immediately with a flow cytometry (Becton Dickinson, USA), and ROS levels were evaluated by the mean green fluorescence intensity.

Measurement of cellular ATP levels

Cellular ATP levels were measured using the ATP Assay Kit (Beyotime, China). ATP lysis buffer was added and centrifuged at 12 000 g for 5 min at 4°C. A 10 μL supernatant was mixed with 100 μL ATP detection working solution and then incubated for 3 min at room temperature, avoiding direct light. The standard substance was prepared to draw the standard curve. The fluorescence of mGCs was measured by a Centro XS3 luminometer (Berthold, Germany). To avoid errors caused by different protein amount, the protein concentration was detected with the BCA Assay Kit (Beyotime, China).

Western blot analysis of OXPHOS complex

Isolated mGCs were lysed in cell lysis buffer (Thermo Fisher Scientific, USA) containing a protease inhibitor cocktail (Roche, Switzerland) for 30 min at 4°C. The cell supernatants were retained at 12 000 *g* for 15 min at 4°C. The bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, USA) was used to determine protein concentrations. Forty micrograms of protein from each sample were separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Invitrogen, USA) and transferred onto polyvinylidene fluoride membranes (Roche, Switzerland) for 1.5 h. After 1 h in blocking buffer containing 5% non-fat dry milk (Becton Dickinson, USA) and 0.1% Tween 20 in Tris-buffered saline (TBS-T), the membranes were incubated overnight at 4°C with anti-OXPHOS (Abcam, UK) and anti-β-Actin (Sigma-Aldrich, USA) antibodies. The membranes were washed three times with TBS-T for 30 min, and incubated with anti-rabbit or anti-mouse secondary antibodies at room temperature for 1 h, and washed three times with TBS-T for 30 min. The protein bands were detected using an enhanced electrochemiluminescence (ECL) Detection System (Thermo Fisher Scientific, USA) and analysed with the Fusion Solo system (Vilber Lourmat, France).

mRNA expression of ATP5A1 and ATP5I genes

mRNA was extracted using a RNA extract Kit (Omega, USA) and reverse transcription was performed using the High Capacity Reverse Transcription cDNA kit (Takara, Japan) according to the manufacturer's instructions, which were determined by qPCR. The primer sequences and reaction conditions are listed in Supplemental Table SII.

Statistical analysis

For the quantitative variables, all data were presented as mean ± SEM. The normal distribution of the values was tested using the Kolmogorov–Smirnov test. The Student t-test was used to analyse these data from two different groups, and linear regression analysis was performed to identify whether items were associated with age. The chi-squared test was used to analyse the qualitative variables. All the calculations were done with SPSS Statistics 17.0 (SPSS, Inc., USA). $P < 0.05$ was considered statistically significant.

Results

Transmission electron microscopy

The nuclei in the mGCs were round or oval, with loose chromatin and some peripheral condensed chromatin. The cytoplasm contained small round lipid droplets and numerous round or elongated mitochondria (Fig. 1A and B). In the young group, most mitochondria were round or oval shaped, with a few intact parallel tubular–vesicular cristae and homogenous matrix density (Fig. 1A), while the mitochondrial shapes in the mGCs from the old group were more heterogeneous, and the elongated mitochondria were mainly observed with numerous cristae and more high-density particles in the matrix (Fig. 1B). Mitochondria with cristae parallel to the long axis were also occasionally observed in the old group (Fig. 1B). The mitochondria that became vacuolus were frequently seen in the mGCs of the older group but occasionally found in the young group (Fig. 1B and C). Swollen mitochondria were also more frequently visible in the older group (Fig. 1D and E).

Quantification of mtDNA copy number and 4977-bp deleted mtDNA

Patients' characteristics are listed in Supplemental Table SIII. The bar chart shows no statistically significant difference between the two groups ($P = 0.194$, Fig. 2). The regression analysis of mtDNA copy number with maternal age suggests that mtDNA relative copy number was positively correlated with maternal age ($r = 0.294$, $R^2 = 0.0867$, $P = 0.009$, Fig. 3). Among the 77 patients, we found no one with 4977-bp deleted mitochondria (data not shown).

Detection of MMP

Patients' characteristics in this part are listed in Supplemental Table SIV. The bar chart shows that the JC-1 ratio of mGCs in the old group was significantly lower than that of the young group (3.01 ± 0.21 vs 3.85 ± 0.27, $P = 0.033$), suggesting more mitochondria with dissipated MMP in the aging group (Fig. 4), and MMP tended to negatively correlate with maternal age ($r = -0.317$, $R^2 = 0.1003$, $P = 0.049$) (Fig. 5). Positive control was shown in the Fig. 6A. Figure 6B shows normal MMP in the young group, while more mitochondria stained green indicates the reduced MMP of mGCs in the aging group (Fig. 6C).

Detection of intracellular ROS levels

Patients' clinical data are listed in Supplemental Table SV. Figure 7 shows that the intensity of ROS detecting fluorescence for mGCs from patients under 38 years old was not significantly different statistically ($P = 0.191$) than patients in the old group.

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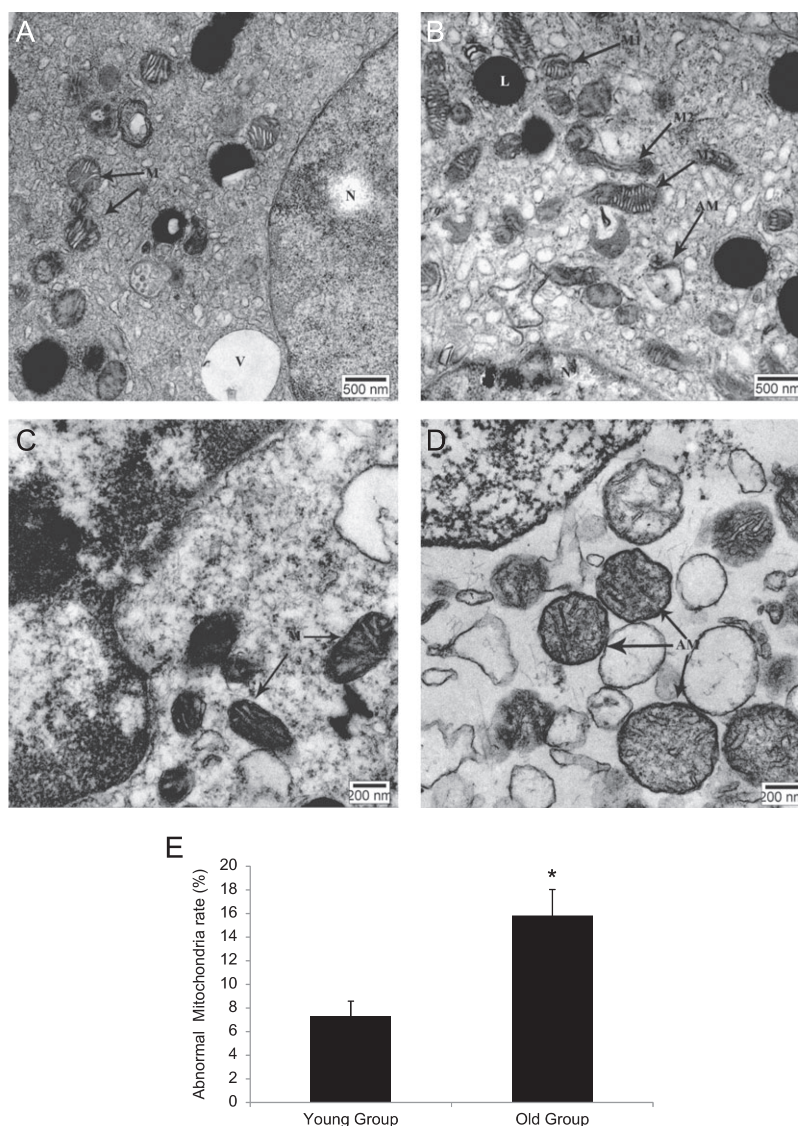

Figure 1 Ultrastructure of mitochondria in the mGCs from the young group and the old group. (**A**) In the young group most mitochondria were round, with a few intact parallel cristae and homogenous matrix density (arrows). (**B**) In the old group mitochondria varying in size and shape were mostly seen (arrows). Among them, there were round shaped mitochondria similar to those in the young group (M1 arrow), and also mitochondria with an elongated shaped, with numerous cristae and high-density granules in the matrix (M3 arrow). Atypical mitochondria with cristae parallel to the long axis were also occasionally observed (M2 arrow). The abnormal mitochondria, like the ones that became vacuolated were often seen (AM arrow). N: nucleus; M: mitochondria; AM: abnormal mitochondria; L: lipid droplets. (**C**) The normal mitochondria with intact parallel cristae in the young group (arrows). (**D**) The swollen mitochondria with membrane almost ruptured and inner cristae broken or absentin the old mGCs group (arrows). (**E**) The rate of abnormal mitochondria in the mGCs from the young and old groups observed by transmission electron microscopy. Abnormal mitochondria were identified according to the damaged ultrastructure, which contained swollen or blurred vacuoles, with ruptured mitochondrial membranes and missing inner cristae. For each mGC sample, a total of 200 mitochondria were counted in at least five random sections and the rate of abnormal mitochondria was recorded. This work was performed by two individuals independently. The bar chart shows that the rate of abnormal mitochondria in mGCs from the patients under 38 years old ($n = 5$) was (7.30 ± 1.28), which was significantly lower than the value (15.80 ± 2.23) of patients at or over 38 years old ($n = 5$), $P = 0.012$. Asterisk represents statistically significant differences ($P < 0.05$).

Measurement of cellular ATP levels

Patients' characteristics in this part are listed in Supplemental Table SVI. Figure 8 shows that the intracellular ATP level of mGCs in the young group was 1.75-fold higher than that of the advanced age group (7.17 ± 1.16 vs 4.15 ± 0.60, $P = 0.025$), which suggested that energy generation declined with maternal aging (Fig. 8).

Western blot analysis of OXPHOS complex

Nine patients under 38 years old and seven patients over 38 years old were analysed after the grey intensity of protein expression was obtained relative to β-actin. We found that protein expression level of ATP5A1, as one of the five proteins of OXPHOS in the young group was higher than that of the old group (1.07 ± 0.13 vs 0.26 ± 0.03,

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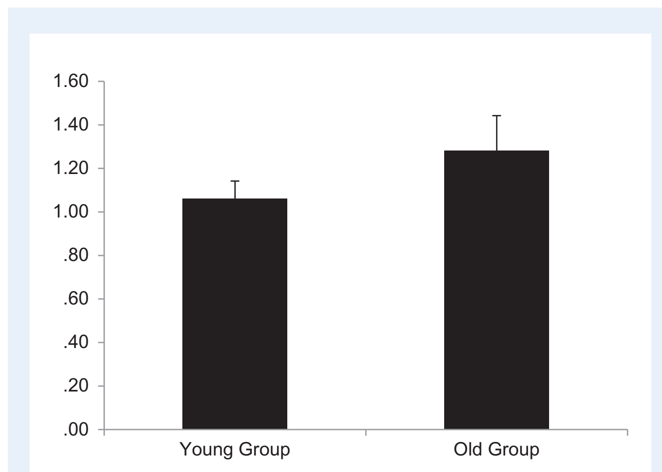

Figure 2 mtDNA relative copy number of the mGCs from the young group and old group detected by the real-time quantitative PCR. The bar chart shows that the mtDNA relative copy number of mGCs from the patients over 38 years old (n = 45) was (1.28 ± 0.16), which was slightly higher than the value (1.06 ± 0.08) of patients under 38 years old (n = 32), but there was no statistically significant difference between the two groups, P = 0.194.

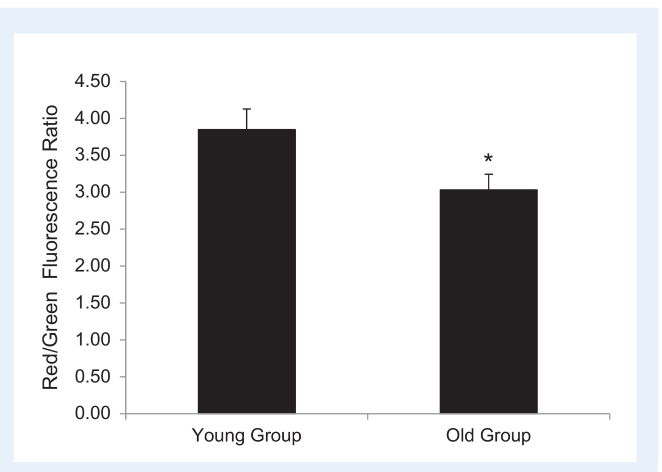

Figure 4 MMP of mGCs from the young group and old group detected by flow cytometry. The bar chart showed that the mean red/green fluorescence intensity ratio for mGCs from the patients under 38 years old (n = 22) was (3.85 ± 0.27), which was significantly higher than the value (3.01 ± 0.21) of patients over 38 years old (n = 17), P = 0.033. Asterisk represents statistically significant differences (P < 0.05).

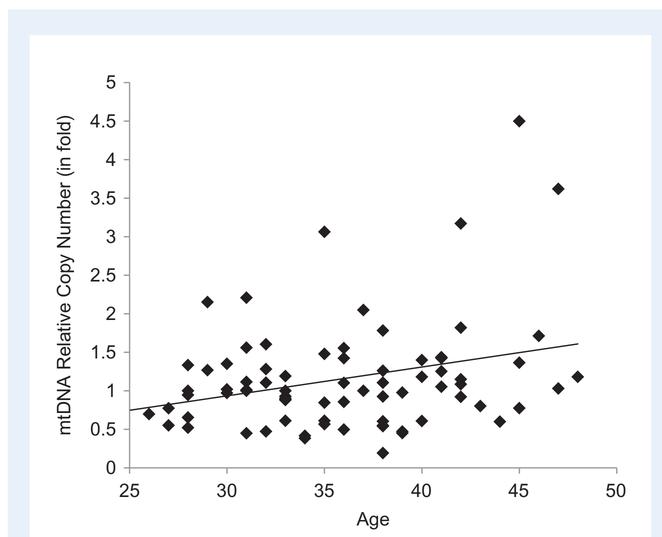

Figure 3 Regression analysis of mtDNA relative copy number with maternal age. The linear correlation between the mtDNA relative copy number in mGCs and age was analysed. The mtDNA relative copy number was associated with maternal age, and the linear equation was (y = 0.0375x − 0.1904), with n = 77, r = 0.294, R^2 = 0.0867 and P = 0.009.

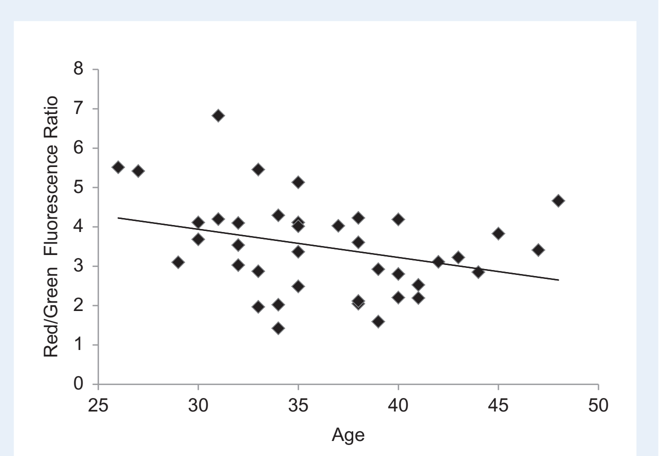

Figure 5 Regression analysis of MMP in the mGCs with age of patients. The linear correlation between the red and green fluorescence ratio in GCs and age was analysed. MMP tended to negatively correlate with maternal age, and the linear equation was (y = −0.0716x + 6.089), with n = 39, r = −0.317, R^2 = 0.1003 and P = 0.049.

P < 0.001, Fig. 9). However, there was no significant difference for the expression levels of other four proteins between the young and old groups (NDUFB8: 0.68 ± 0.12 vs 0.39 ± 0.06, P = 0.079, SDHB: 1.23 ± 0.32 vs 0.86 ± 0.23, P = 0.386, UQCRC2: 0.69 ± 0.08 vs 0.51 ± 0.08, P = 0.128, MTCO1: 1.21 ± 0.39 vs 1.00 ± 0.30, P = 0.687).

mRNA expression quantification of ATP5A1 and ATP5I genes

Characteristics of fifty-three patients are shown in Supplemental Table SVII. The regression analysis suggested that ATP5A1 mRNA expression was negatively correlated with maternal age (r = −0.341, R^2 = 0.1163, P = 0.012, Fig. 10). There was no statistically significant association between ATP5I mRNA expression and age (r = −0.254, R^2 = 0.0645, P = 0.066, Fig. 11).

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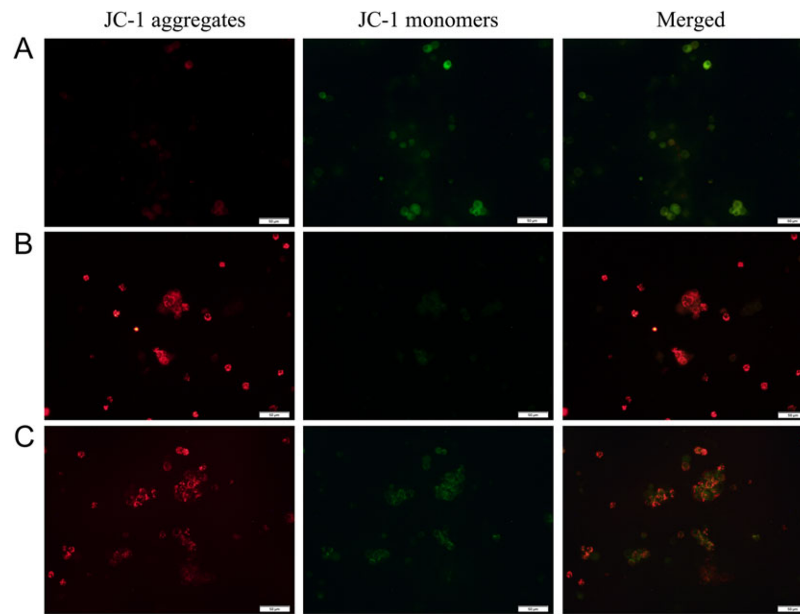

Figure 6 MMP observed with fluorescent microscope in mGCs from the positive control group, the young group and the old group respectively. (**A**) Cells containing JC-1 monomers had low MMP and show green fluorescence, while JC-1 aggregates showing red fluorescence revealed high MMP. mGCs were incubated with CCCP (carbonyl cyanide 3-chlorophenylhydrazone) (10 μM) for 20 min and then stained with JC-1, as described in Materials and Methods. MMP of mGCs were completely disrupted after exposure to CCCP, and only green fluorescence can be seen. Thus, this group was regarded as the positive control. (**B**) Both red and green fluorescence were observed in the mGCs from the young group after staining with JC-1. (**C**) The intensity of red fluorescence excited in the mGCs of the older group were weaker when compared with the young group while an increase of green fluorescence was seen in the old group. Scale bar 50 μm.

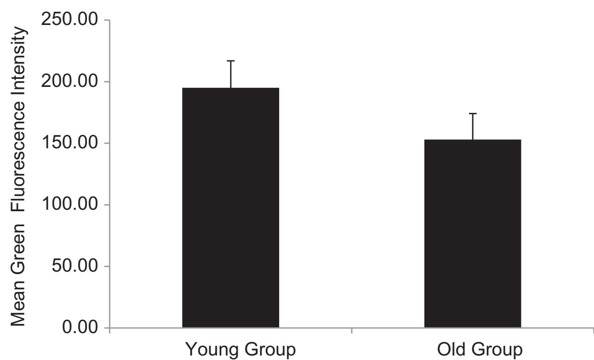

Figure 7 Intracellular reactive oxygen species (ROS) levels of the mGCs from the young group and old group detected by flow cytometry. The bar chart showed that the mean green fluorescence intensity for mGCs from the patients under 38 years old ($n = 24$) was (195.00 ± 21.92), which was slightly higher than the value (153.00 ± 21.65) of patients over 38 years old ($n = 17$), but the analysis indicated that there was no statistically significant difference in terms of intracellular ROS levels between two groups, $P = 0.191$.

Discussion

In the present study, we focused on the mitochondria in mGCs and found age-related changes of mitochondrial ultrastructure, accompanied by decreased MMP in the old group. However, changes of intracellular ROS level, mtDNA content and its integrity did not decline significantly with aging. Finally, reduced ATP production and impaired ability of OXPHOS were observed in the study, which may be mainly responsible for age-related dysfunction of mGCs.

In our study, mitochondria in the young group were mostly round with intact parallel cristae, while elongated mitochondria with numerous cristae were more common in the old group. In previous study, Fair et al. (1997) reported that round mitochondria were dominant in primordial follicles, which does not require a large amount of energy, while secondary follicles contain a larger number of elongated mitochondria, which means that elongated mitochondria are representative in mature forms to produce ATP. Our results suggested mitochondria from advanced age women were more differentiated. Thus, with maternal aging, these compensatory morphological changes can be used to explain the decrease of their physiological function. Another important age-related change was the increase of the atypical or abnormal mitochondria in the old group. Mitochondria with longitudinally

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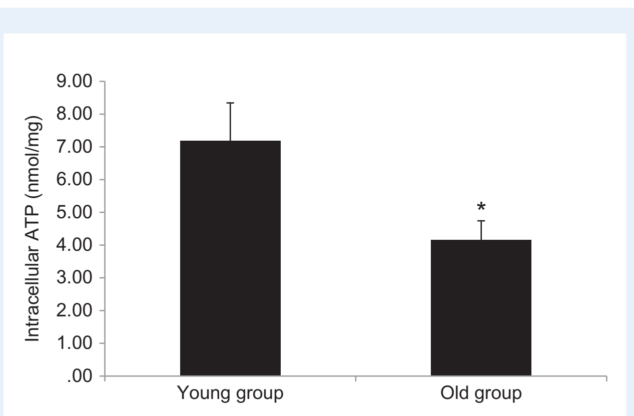

Figure 8 Intracellular ATP level in mGCs of the young group and old group. The bar chart showed that the mean intracellular ATP level for mGCs from the patients under 38 years old ($n = 33$) was (7.17 ± 1.16), which was significantly higher than the value (4.15 ± 0.60) of patients over 38 years old ($n = 22$), $P = 0.025$. Asterisk represents statistically significant differences ($P < 0.05$).

oriented cristae were also observed in human co-cultured granulosa cells (Motta et al., 1995), which may be caused by high oxygen concentration or aging (Bhardwaj and Saraf, 2014; de Bruin et al., 2004; Krockova et al., 2013).

mtDNA was analysed to evaluate the amount of mitochondria, and evidence suggests that both quality and quantity of mtDNA are associated with IVF outcomes (Ogino et al., 2016; Seifer et al., 2002; Wang et al., 2010). However, there have been few investigations about the age-related changes of mtDNA. Our results showed that the mtDNA relative copy number in the older group was not significantly different statistically than in the younger group. Although the regression analysis suggested an association between mtDNA copy number and age, while intracellular ATP production decreased with maternal age in our study. There may be a compensatory phenomenon that more mitochondria are replicated to satisfy the normal energy demand once the mitochondria are impaired. This is supported by the observation that an increased amount of mtDNA in euploid embryos is related to poor implantation potential (Diez-Juan et al., 2015). Mitochondria isolated from patient's own mGCs have also been used to supply oocytes and

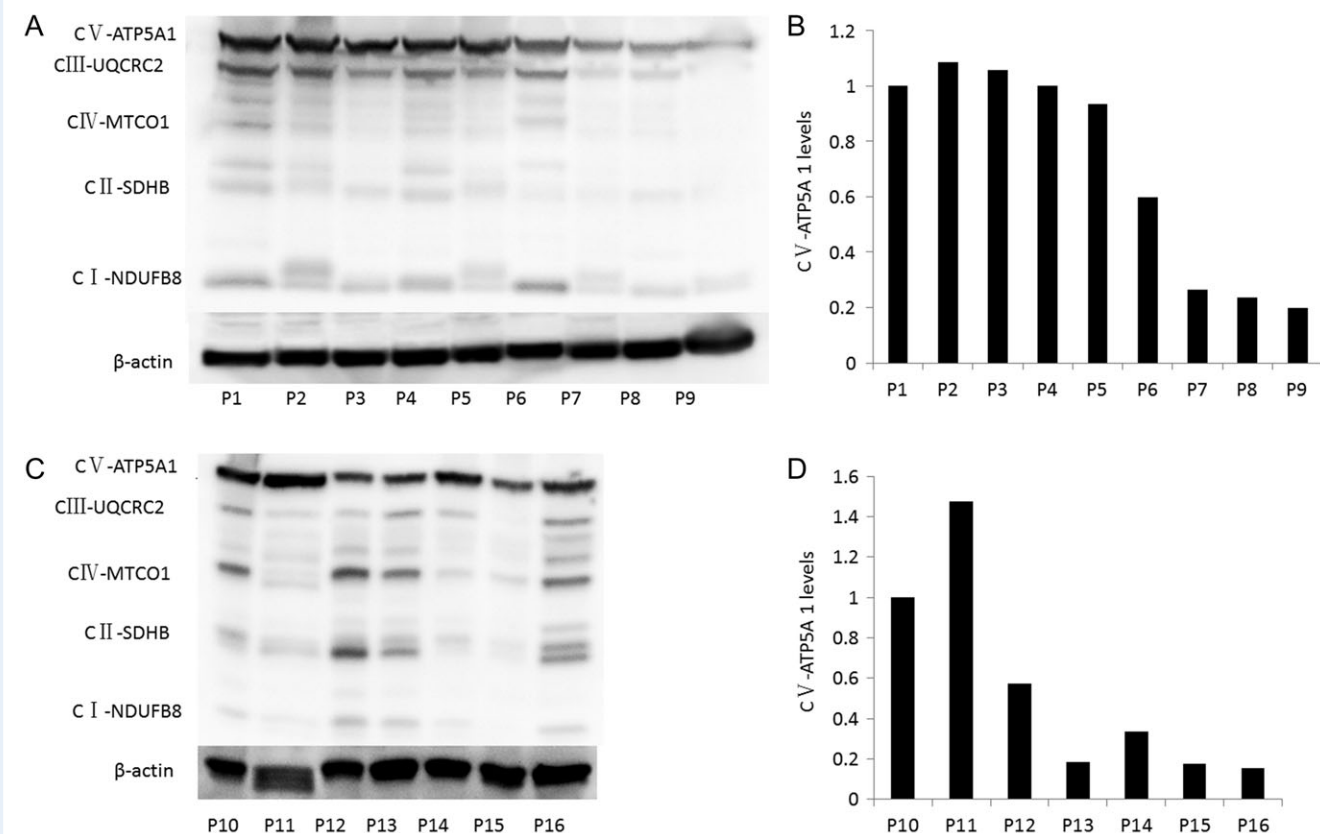

Figure 9 Mitochondrial function of oxidative phosphorylation (OXPHOS) in mGCs impaired with maternal aging. (**A**) Expression of OXPHOS subunits, including NDUFB8 (Complex I), SDHB (Complex II), UQCRC2 (Complex III), MTCO1 (Complex IV) and ATP5A1 (Complex V) were detected by Western blot analysis of nine patients with different ages (P1: 28 y, P2: 27 y, P3: 23 y, P4: 30 y, P5: 33 y, P6: 35 y, P7: 41 y, P8: 42 y, P9: 44 y). (**B**) The densitometry of the ATP5A1 protein staining for nine patients, relative to β-actin. (**C**) Expression of OXPHOS subunits were detected by Western blot analysis of seven patients from the young and old groups (P10: 28 y, P11: 29 y, P12: 28 y, P13: 42 y, P14: 49 y, P15: 48 y, P16: 40 y). 30 μg of protein lysate obtained from mGCs were separated on 12% SDS-PAGE, and protein loads were evaluated by β-actin antibody staining in panels A and B which suggested the equal loading quantity of protein sample in different patients. (**D**) The densitometry of the ATP5A1 protein staining for seven patients, relative to β-actin.

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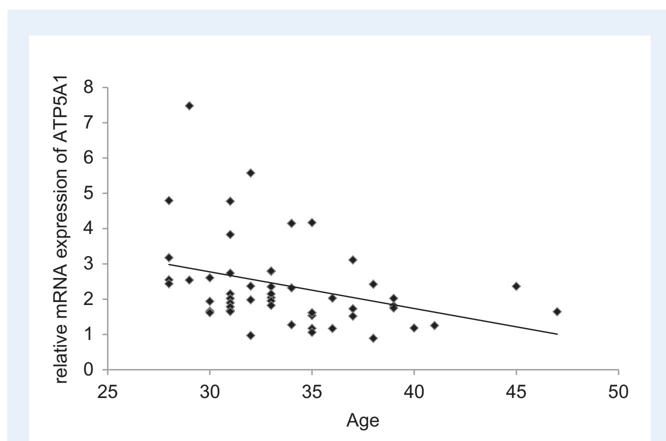

Figure 10 Regression analysis of ATP5A1 mRNA expression in the mGCs with maternal age. The linear correlation between ATP5A1 mRNA expression in GCs and age was analysed. The mRNA expression of ATP5A1 tended to negatively correlate with maternal age, and the linear equation was ($y = -0.104x + 5.8932$), with $n = 53$, $r = -0.341$, $R^2 = 0.1163$ and $P = 0.012$.

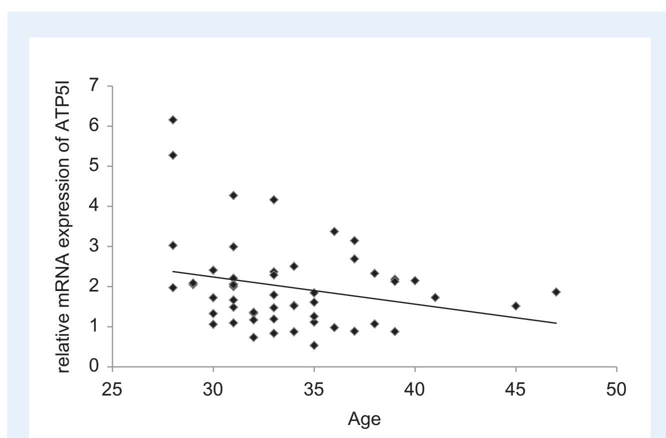

Figure 11 Regression analysis of ATP5I mRNA expression in the mGCs with maternal age. The linear correlation between ATP5I mRNA expression in GCs and age was analysed. The mRNA expression of ATP5I tended to decrease with maternal age, but the correlation was not statistically significant. The linear equation was ($y = -0.0678x + 4.2739$), with $n = 53$, $r = -0.254$, $R^2 = 0.0645$ and $P = 0.066$.

improve IVF outcomes (Tzeng et al., 2001). We believe that the feasibility of mitochondrial transplantation requires further investigation, as it remains unclear whether mitochondrial dysfunction or lack of mtDNA is the root cause.

The 4977-bp deletion of mitochondrial DNA has been reported in aged human central nervous systems, blood cells and mGCs (Seifer et al., 2002; Zabihi Diba et al., 2016). In the present study, no one was identified with mitochondrial DNA 4977-bp deletion. Our findings are in accordance with a previous study (Tan and Li, 2010).

MMP, an important marker of mitochondrial activity, generally decreases in response to oxidative stress (Lemasters et al., 1999). Our results showed that MMP was significantly higher in the young group, and negatively correlated with age. Considering the decreased MMP of mGCs and reduced numbers of total or mature oocytes retrieved in the aging group, we suggest that mGCs involve energy facilitating oocyte development. Our findings are in accordance with the conclusions on CCs from Dumesic et al. (2016).

Although increased intracellular ROS levels are believed to be linked with the loss of metabolic energy homoeostasis during aging, studies of ROS in mGCs are still scant (Gonzalez-Fernandez et al., 2016). In our study, intracellular ROS levels were detected, and no difference was found between the groups. This may be explained by the decreased OXPHOS function with age described in our results because ROS is mainly generated along with ATP. Therefore, antioxidant defense mechanisms could be combined to evaluate the cellular oxidative damage with aging. This is supported by a report, which showed that ROS levels were not different in young and old mice oocytes under baseline conditions, but significantly increased in the old group after exposure to H_2O_2 (Babayev et al., 2016).

Intracellular ATP levels are often detected as a critical parameter to study mitochondrial function, particularly, the ability of OXPHOS. The idea that mitochondrial OXPHOS in mGCs is linked with reproductive aging remains controversial. Some investigators support that, unlike the oocytes, mitochondria in mGCs are not affected by aging or the diminishment of the ovarian response to gonadotropins (Shufaro et al., 2012). We hypothesized that intracellular ATP levels would reduce with aging and finally restrict mitochondrial function in mGCs. The intracellular ATP level of mGCs in the young group was 1.75-fold higher than that of the advanced age group, which supported the idea that energy generation declined in advanced mGCs, accompanied by western blot analysis of OXPHOS complex proteins in this study. Previous studies on the relationship between OXPHOS and reproductive age in mGCs have yielded different conclusions. Ben-Meir et al. found that mitochondrial complex activity in the reproductive system reduced with aging and improved with CoQ10 supplementation (Ben-Meir et al., 2015). A study revealed that expressions of ATP5I in CCs decreased with aging at both the transcript and protein levels (McReynolds et al., 2012). However, the report suggested that the function of the respiratory chain in mitochondria of mGCs, more specifically, the activity of cytochrome *c* oxidase and succinate dehydrogenase was unaffected by age or ovarian response (Shufaro et al., 2012). In our study, complex ATP5A1 decreased with aging, while there were no differences in other complex proteins and ATP5I mRNA expression with aging. This was the first report to suggest that ATP5A1 plays a key role in the mitochondria dysfunction of mGCs with maternal aging.

In conclusion, one of the highlights of this study is that mGC dysfunction with maternal aging is mainly linked to the impaired mitochondrial functions, especially for OXPHOS function, rather than increased intracellular ROS levels, reduced mitochondrial content or deletions of mtDNA. Our study suggested that increasing the ability of OXPHOS in mGCs may improve infertility in advanced age women undergoing ART. Meanwhile, the feasibility of autologous mitochondria transplantation from mGCs is doubtful because just supplementing dysfunctional mitochondria does not work. The small quantity of mGCs in individual patients and the short survival time of mGCs cultured *in vitro* limit further study of the mechanism, and a replaceable cell model should be built to solve this problem. The root cause of reduced OXPHOS ability in aging and how to improve it still need further research.

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Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors' roles

Y.Y. and Y.L. take primary responsibility for the study concept and design. Y.L. and M.H. quantified the mtDNA content, 4977-bp deleted DNA and mRNA expression of ATP5A1 and ATP 5I and performed western blot and detection of ATP. Y.L. and M.H. analysed the data and drafted the article. Y.L. and X.L. collected samples and performed the detection of TEM, ROS and MMP. H.W., M.M. and S.Z. recruited the patients. Y.G. and S.W. collected patients' clinical data. Y.W., N.D., B.X. and J.Y. revised the article. All of the authors reviewed the article prior to submission. The two lead authors are co-first authors, making an equal contribution.

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Conflict of interest

None declared.

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