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Mitochondrial aggregation patterns and activity in human oocytes and preimplantation embryos

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Mitochondria play a vital role in the metabolism of energy-containing compounds in the oocyte cytoplasm to provide adenosine trisphosphate for fertilization and preimplantation embryo development. In this study, ratiometric confocal microscopy with the mitochondrion-specific membrane potential-sensitive fluorescence dye JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide) was used to measure the activity of mitochondria in human oocytes and developing preimplantation embryos. Mitochondria in oocytes and embryos were characterized by distinct localized aggregation patterns. These patterns however did not determine localized regions of heterogeneity in mitochondrial activity. Mitochondrial activity was analysed during oocyte maturation and after fertilization. The activity of mitochondria in fresh metaphase II oocytes was negatively correlated with maternal age. This trend continued when the activity of developing embryos was analysed. Mitochondrial activity was strongly correlated with the rate of embryo development on day 3 after fertilization, but not on day 2. Partial regression analysis showed that the rate of cleavage of preimplantation embryos was more highly correlated with embryo mitochondrial activity than maternal age. These data suggest that the efficiency of mitochondrial respiration in oocytes and preimplantation embryos is closely correlated with the programmed rate of embryo development, and suggest that maternal age further influences this factor. The loss of mitochondrial activity in oocytes obtained from ageing couples may therefore contribute to lower embryo development and pregnancy rates observed during cycles of IVF.

Key words: cell cycle/cellular metabolism/IVF/mitochondria/oocytes

Introduction

In all eukaryotes, mitochondria are specialized organelles that catalyse the formation of adenosine trisphosphate (ATP) through the metabolism of carbohydrates and fats contained within the cell cytoplasm and the external medium. Major mitochondrial disorders can lead to human diseases such as premature ageing, myopathies and neurodegenerative disease (see Larsson and Clayton, 1995). Minor mitochondrial disorders can lead to infertility (Folgero *et al*., 1993; Reynier *et al*., 1998; Troiano *et al*., 1998).

The rate of cellular respiration depends on two major factors. The first factor is the efficiency of conversion in the oocyte cytoplasm of metabolic precursors such as glucose to pyruvate (Alberts *et al*., 1983). The second factor is the efficiency of the mitochondrial matrix in the conversion of pyruvate to ATP, the latter being required for diverse cellular processes in developing embryos, including cell division, DNA replication and genomic activation. The metabolism of mammalian oocytes is specialized in that pyruvate is the major utilizable energy source (Biggers *et al*., 1967; Bavister, 1995; Gardner, 1998). The addition of pyruvate into in-vitro human embryo culture medium therefore reduces the significance of cytoplasmic metabolic enzymes in the supply of metabolic precursors during preimplantation embryo development. This leaves the efficiency of mitochondrial respiration as the major factor involved in the production of ATP during this stage.

Human preimplantation embryos are characterized by diverse rates of development and potential for implantation. Although genetic and paternal factors may contribute to these differences (Janny and Ménézo, 1994; Ménézo and Dale, 1995; Warner *et al*., 1998), one of the major factors that influences the development and implantation rate of human embryos is maternal age (Cummins *et al*., 1994; Janny and Ménézo, 1996; Keefe, 1997). These data suggest that embryos from older patients have accumulated negative factors lowering the implantation rate (Cummins *et al*., 1994). The factors may include errors in the efficiency of ATP production through mitochondrial respiration (Van Blerkom *et al*., 1995). This hypothesis is supported by the fact that the transfer of cytoplasm

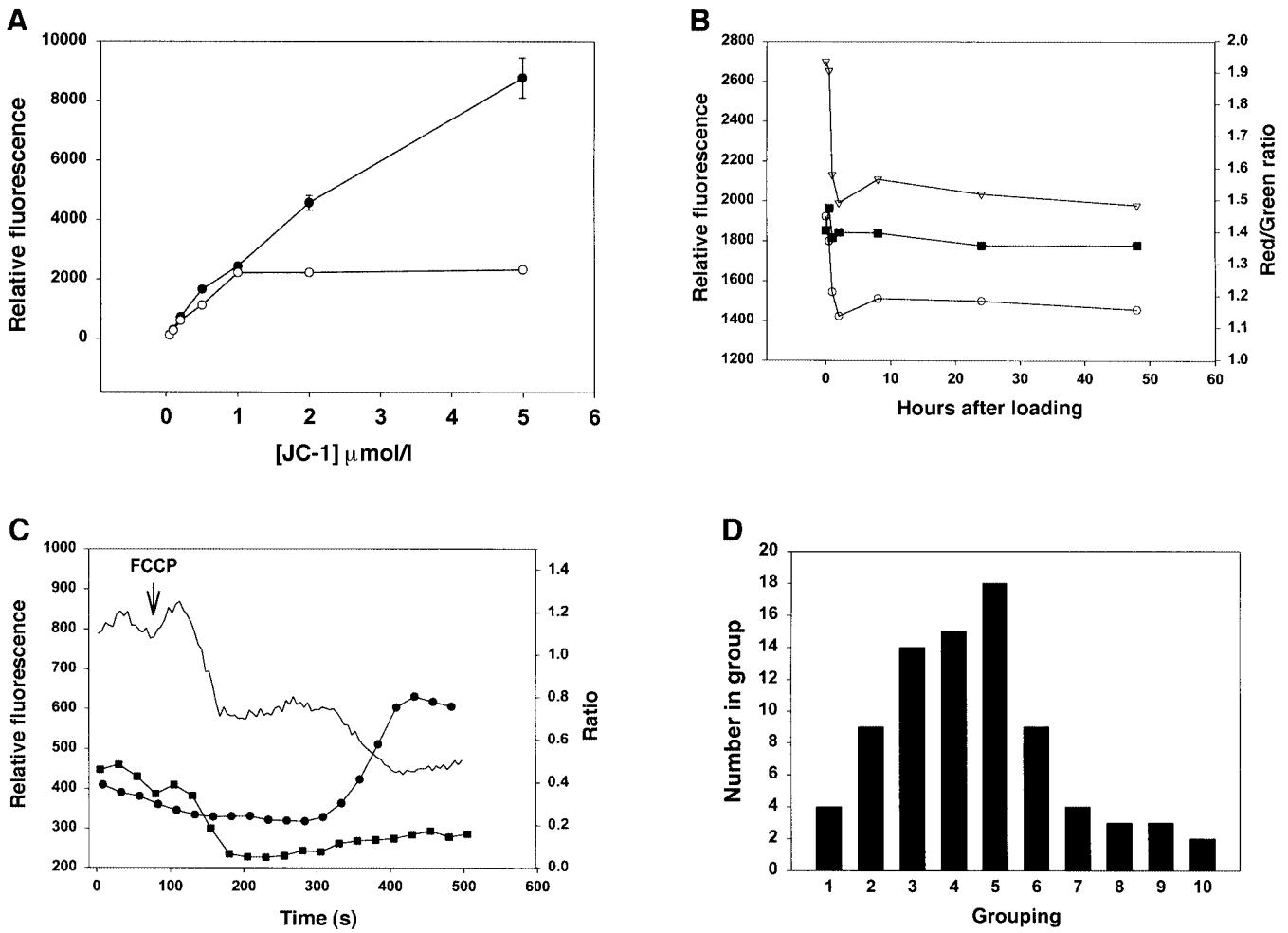


Figure 1. Characteristics of JC-1 loading in human oocytes. (**A**) Saturation curve of JC-1 loading in human oocytes. Oocytes were loaded with a range of concentrations of JC-1. After 30 min, the confocal fluorescence was obtained. ●, green fluorescence; ○, red fluorescence. Points are shown as mean and SE bars. Five measurements were taken using failed-to-fertilize oocytes for each concentration. From the curve, it is calculated that 0.5 μmol/l JC-1 permits a linear relationship between concentration and fluorescence ratio. (**B**) Oocytes were loaded for 30 min with 0.5 μmol/l JC-1. The fluorescence ratio was recorded immediately after loading and at defined intervals. The data relate to a representative experiment from four examples. ▽, relative green fluorescence; ○, red fluorescence (left axis); ■, ratio obtained by division of the red fluorescence by green fluorescence (right axis). (**C**) Fresh metaphase II oocytes were scanned at 5-s intervals after loading with 0.5 μmol/l JC-1. FCCP [carbonylcyanide *p*-(trifluoromethyl)phenylhydrazone] was added to the bath to a final concentration of 5 μmol. The experiment is representative of a total of six separate experiments. ●, green fluorescence, ■, red fluorescence channel. The continuous line is the ratio of red/green fluorescence. The left axis is the axis for the raw fluorescence, whereas the right axis numerates the ratio. (**D**) Fresh metaphase II oocytes were scanned through the region of the polar body after loading with 0.5 μmol/l JC-1. The fluorescence readings from the green confocal emission channel were grouped into bins of relative intensity/μm^2. The data show the results of a total of 81 readings and follow the normal distribution (Kolmogorov–Smirnov test = 0.259, $P = 0.05$).

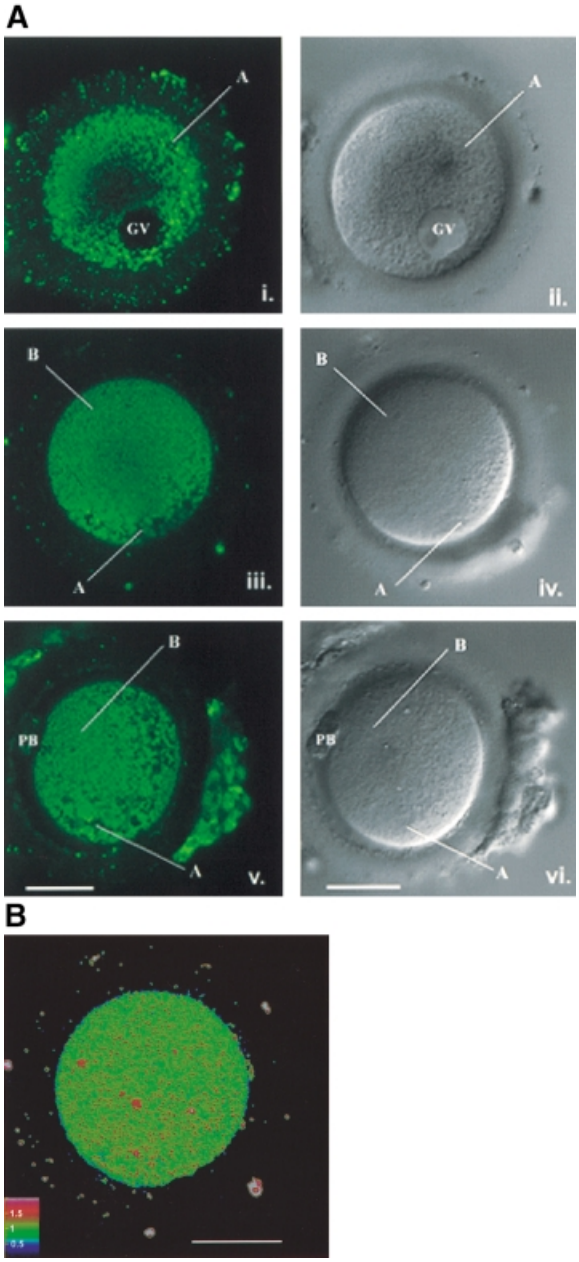
between mouse oocytes increased ATP production in recipients (Van Blerkom *et al.*, 1998) and may improve the development and implantation rate in human embryos produced after IVF (Cohen *et al.*, 1998).

JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) is a fluorescent dye that accumulates in mitochondria and reports the membrane potential across the matrix membrane (Reers *et al.*, 1991, 1995). Because this value is highly related to mitochondrial respiratory rate, JC-1 can be used as an indicator of mitochondrial activity. In this study, JC-1 and ratiometric confocal microscopy were used to monitor the activity of mitochondria in human oocytes and preimplantation embryos. The results suggest that mitochondrial activity is highly correlated with maternal age, and that this is the cause of the slower development rate of embryos observed in these patients.

Materials and methods

Patients

Patients were attending IVF clinics for IVF protocols. Spare oocytes and embryos not suitable for transfer or cryopreservation were donated for research after informed consent. Fresh human oocytes were obtained after informed consent in cases where the donation of these oocytes to the research programme would have little effect on the outcome of the IVF cycle. Patients were prepared for IVF using standard ovarian stimulation protocols including down-regulation of the pituitary gland with a gonadotrophin-releasing hormone (GnRH)



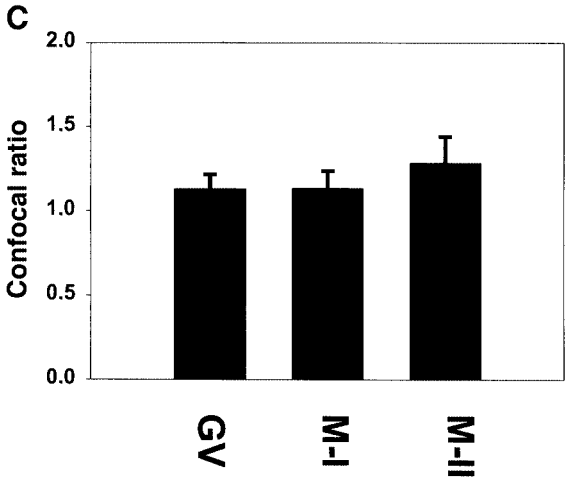


Figure 2. Mitochondrial aggregation patterns and activity during oocyte maturation. (**A**) Two distinct types of mitochondrial aggregation pattern during oocyte maturation. Images on the left are fluorescence images from the potential-insensitive confocal channel; images on the right are transmitted light images taken simultaneously during confocal scans. (i., ii.): images of a germinal vesicle-stage oocyte representative of a total of six; (iii., iv.): images from metaphase I-stage oocytes representative of a total of four oocytes. (v., vi.): metaphase II-stage oocytes representative of a total of 81 oocytes imaged. 'A' delineates mitochondrial aggregation pattern type A; 'B' indicates mitochondrial aggregation pattern type B. GV = germinal vesicle; PB = polar body. Scale bar = 50 μm. (**B**) Homogeneity of mitochondrial activity in oocytes. Confocal images from the red channel were divided pixel-by-pixel by images from the green channel; the result is a map of mitochondrial activity. The colour bar shows the ratio obtained. The image is a representative example of a total of 81 oocytes. Scale bar = 50 μm. (**C**) Mitochondrial activity during oocyte maturation. Confocal ratios were taken for oocytes at various stages of maturation. Bars indicate SE of the mean for germinal vesicle-stage oocytes (n = 6); for metaphase I (M-I) -stage maturing oocytes (n = 4); and for metaphase II (M-II) -stage oocytes (n = 81). No significant differences were observed between any of the three stages of maturation.

Table I. Observations of mitochondrial morphology during oocyte maturation

Stage	Oocytes with		
	type A only (%)	type B only (%)	type A and B (%)
GV	5/6 (83)	1/6 (17)	0/6 (0)
M I	0/4 (0)	1/4 (25)	3/4 (75)
M II fresh	0/81 (0)	21/81 (26)	60/81 (74)
M II aged	0/30 (0)	29/30 (97)	1/30 (3)

GV = germinal vesicle-stage immature oocytes; M I = metaphase I-stage immature oocytes; M II = metaphase II-stage oocytes.

agonist (Decapeptyl; Ibsen, Italy) followed by ovarian stimulation with exogenous FSH. Oocyte retrieval was performed 36 h after the administration of 10 000 IU human chorionic gonadotrophin (β-HCG) when two to three follicles of 18–20 mm diameter were observed by ultrasound examination, and blood 17β-oestradiol concentrations reached 150–200 pg/ml per follicle over 18 mm. Oocytes were processed for standard IVF and intracytoplasmic sperm injection (ICSI) protocols using commercial IVF medium (Medicult, Copenhagen, Denmark) pre-equilibrated to 37°C and 5% CO_2. Human preimplantation embryos were obtained on day 2 or 3 after fertilization, after the completion of embryo transfer or cryopreservation. The embryos were graded morphologically, and subsequently re-examined immediately prior to the experiment.

Fluorescence labelling and confocal microscopy

The potential-sensitive fluorescence dye JC-1 (Molecular Probes, Eugene, Oregon, USA) was used to measure the activity of mitochondria (Reers *et al*., 1991, 1995). The dye was dissolved to a stock concentration of 0.5 mmol/l in dimethylsulphoxide and diluted into pre-equilibrated IVF medium (Medicult), using a vortex to aid the dissolution of the dye, as required. Under these conditions, the dye

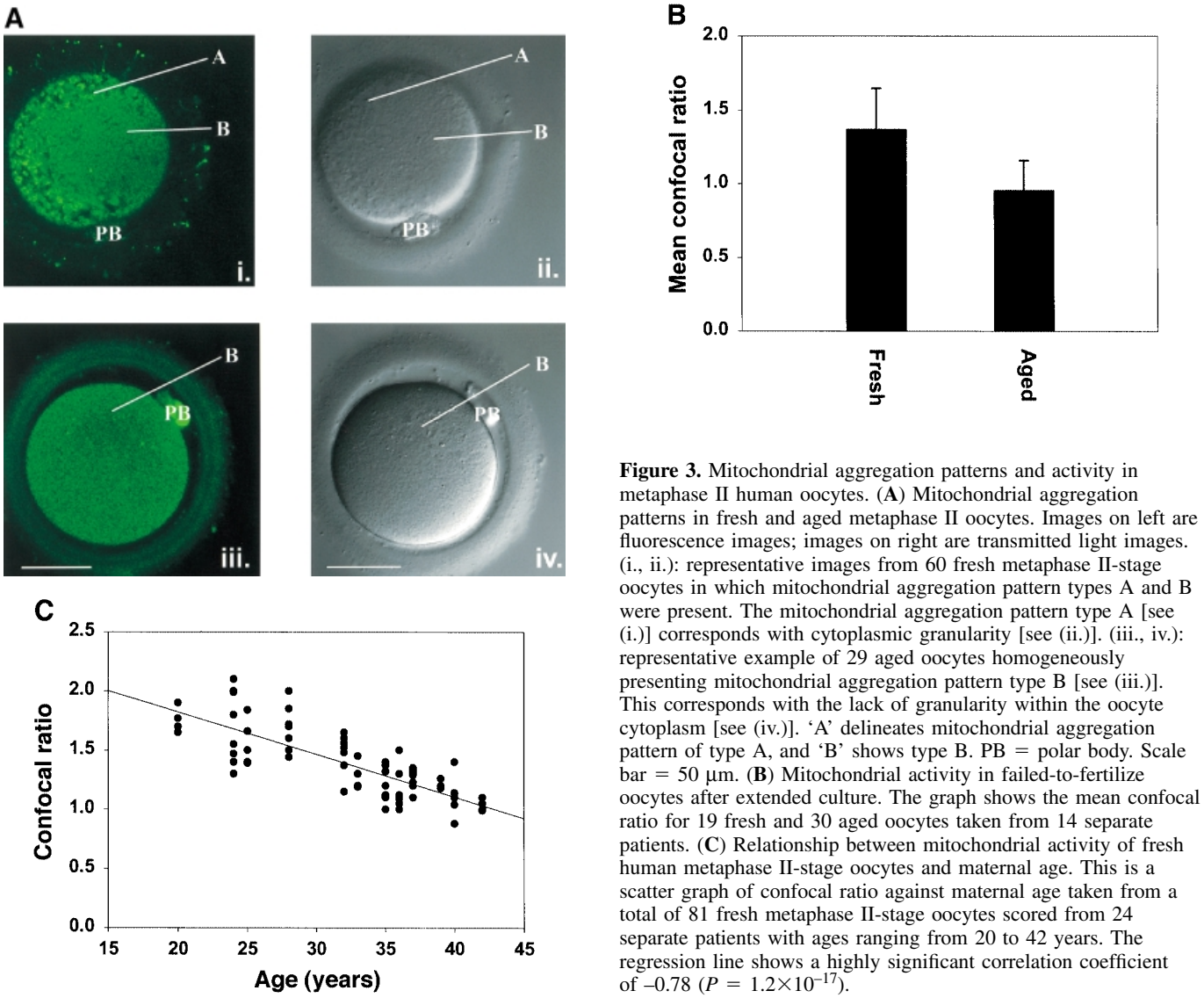


Figure 3. Mitochondrial aggregation patterns and activity in metaphase II human oocytes. (**A**) Mitochondrial aggregation patterns in fresh and aged metaphase II oocytes. Images on left are fluorescence images; images on right are transmitted light images. (i., ii.): representative images from 60 fresh metaphase II-stage oocytes in which mitochondrial aggregation pattern types A and B were present. The mitochondrial aggregation pattern type A [see (i.)] corresponds with cytoplasmic granularity [see (ii.)]. (iii., iv.): representative example of 29 aged oocytes homogeneously presenting mitochondrial aggregation pattern type B [see (iii.)]. This corresponds with the lack of granularity within the oocyte cytoplasm [see (iv.)]. 'A' delineates mitochondrial aggregation pattern of type A, and 'B' shows type B. PB = polar body. Scale bar = 50 μm. (**B**) Mitochondrial activity in failed-to-fertilize oocytes after extended culture. The graph shows the mean confocal ratio for 19 fresh and 30 aged oocytes taken from 14 separate patients. (**C**) Relationship between mitochondrial activity of fresh human metaphase II-stage oocytes and maternal age. This is a scatter graph of confocal ratio against maternal age taken from a total of 81 fresh metaphase II-stage oocytes scored from 24 separate patients with ages ranging from 20 to 42 years. The regression line shows a highly significant correlation coefficient of –0.78 ($P = 1.2\times10^{-17}$).

was found to remain dissolved for up to 1 h, permitting accurate loading of oocytes. An Olympus Fluoview (Olympus, Segrate, Italy) confocal microscope, based on an Olympus IX-70 inverted microscope, was used for all experiments. A Kr/Ar laser was used to produce the excitation laser line at 488 nm, and emission wavelengths were separated by a 530 nm dichroic mirror followed by analysis in a photomultiplier after further filtering through a 515–530 nm bandpass filter (green emission) or a 585 nm longpass filter (red emission). Laser power and photomultiplier settings were kept constant for all experiments. Oocytes were positioned with the polar body in the plane of focus where present, and a single scan through the centre of the oocyte was used for the analysis. For embryos, single scans through the centre of focus of each blastomere were used for the analysis. Areas of embryo fragmentation and overlaps between blastomeres were excluded from the analysis by deselecting then with the confocal software. Aged oocytes were scored as unfertilized on day 1 after cycles of IVF. Controls for this group were fresh oocytes analysed on the day of oocyte retrieval, donated by the same patient as the aged material. Oocytes and embryos were used only once before being discarded. Images were processed by the confocal software and Adobe Photoshop.

Statistical analysis

All data were plotted as mean ± SD unless stated otherwise. All plots and statistical analysis were calculated using the Sigma Plot and Sigma Stat software packages [Statistics Package for Social Sciences (SPSS), Erkrath, Germany] except where stated. The strength of the normal distribution was tested using the Kolmogorov–Smirnov normality test. Regression lines were calculated by the method of least squares, and the significance of the regression lines was tested with the Pearson product-moment test. Partial regression analysis was calculated using the SPSS statistical package. The Mann–Whitney rank sum test was used to adjust the *t*-test for small populations of data.

Results

The use of JC-1 to measure mitochondrial membrane potential

JC-1 is a dual emission, potential-sensitive dye that accumulates preferentially within mitochondria in the oocyte cytoplasm (Reers *et al.*, 1991, 1995). Because the major movement of

Table II. Relationship between mitochondrial activity and maternal age in unfertilized oocytes

	Maternal age (years)				
	20–24	25–29	30–34	35–39	>40
No. of patients	3	3	5	10	3
No. of oocytes	17	10	19	36	3
Confocal ratio[a]	1.7 ± 0.2	1.6 ± 0.2	1.3 ± 0.2	1.2 ± 0.1	1.1 ± 0.1
95% CI	0.09	0.04	0.1	0.1	0.1

[a]Values are mean ± SD.
CI = confidence interval.

charge across mitochondrial membranes in a steady state is carried by hydrogen ions produced during cellular respiration (Alberts *et al*., 1983), the dye can be used to measure the rate of respiration in individual oocytes and blastomeres of preimplantation embryos. The sensitivity of JC-1 to membrane potential is characterized by an increase in red fluorescence emission (Reers *et al*., 1991, 1995). However, because the fluorescence ratio is dependent on dye concentration (Reers *et al*., 1991, 1995), it was first necessary to establish a dye loading protocol that measured charge movement independent of dye concentration artefacts. Individual oocytes were loaded for 30 min with different stock concentrations of JC-1, and the fluorescence ratio measured. The results showed saturation of red fluorescence at 1 μmol/l, with a K_m of 0.5 μmol/l dye (Figure 1A). Thus, a loading concentration of 0.5 μmol/l was used for all further experiments. JC-1 initially distributes within the oocyte cytoplasm in a concentration- and charge-dependent manner (Reers *et al*., 1991, 1995). To ensure that the final distribution of JC-1 was independent of excess dye within the oocyte cytoplasm, the dye ratio was measured at different times after completion of loading. The results showed that the ratio of red to green fluorescence stabilizes 60 min after dye loading (Figure 1B). The residual dye within the oocyte cytoplasm was confirmed to be concentration-independent by incubating oocytes up to 48 h with no further dye loading; the fluorescence of the green emission channel did not significantly change during this incubation (Figure 1B). These data demonstrate that the distribution of dye under such conditions is uniquely dependent on the charge distribution within the oocyte mitochondria. To confirm further that the JC-1 fluorescence ratio accurately measured mitochondrial membrane potential, the mitochondrial uncoupler carbonyl cyanide *p*-(trifluoromethyl) phenylhydrazone (FCCP; Sigma, Milan, Italy) was used to eliminate the mitochondrial membrane potential. The addition of FCCP to the unfertilized oocyte caused rapid destruction of the fluorescence ratio (Figure 1C). These data demonstrate that the dye JC-1 can accurately report mitochondrial membrane potential in human oocytes.

In order to compare accurately the diverse populations of oocytes analysed on different days, it was necessary to establish whether the dye loading protocol was reliable in terms of the concentration of dye in the oocyte cytosol. This was tested by examining the fluorescence output of the green confocal channel in oocytes used on different days, previous data having demonstrated that this value remains relatively constant for a range of mitochondrial membrane potentials (Reers *et al*., 1991). Under the present loading conditions, it was found that the green fluorescence intensity for standard confocal settings followed the normal distribution with a relative intensity of 4.12 ± 2.20 (n = 81; Kolmogorov–Smirnov distribution 0.259, P = 0.05; Figure 1D). It was therefore concluded that the dye loading protocol, when used on consecutive days, was accurate at the 95% level.

Human oocyte maturation is characterized by changes in mitochondrial aggregation pattern and not mitochondrial membrane potential

The fluorescence dye JC-1 can be used to determine both the activity and localization of mitochondria in human oocytes and preimplantation embryos. First, the localization of mitochondria in human oocytes was determined at various stages of maturation by analysing data from the potential-insensitive confocal channel. Germinal vesicle-stage oocytes obtained on the day of oocyte retrieval were characterized by mitochondria with a granular, clumped aggregation pattern which was termed type A and which coincided with the appearance of the oocyte under Nomarski optics (Figure 2A; Table I). Maturation of oocytes to metaphase I or II led to the appearance of a second type of pattern, the appearance of which was smooth under both fluorescence and light microscopy (Figure 2A). This was termed mitochondrial pattern type B. Both metaphase I and II oocytes had a mixture of granular (type A) and smooth mitochondria and in consequence cytoplasm (type B, Figure 2A; Table I). These data demonstrate that the mitochondrial aggregation pattern in the oocyte cytoplasm is directly related to the appearance of the oocyte under the light microscope, and further suggest that mitochondrial aggregation is modified during oocyte maturation. Localized areas of heterogeneity in mitochondrial membrane potential have been reported in other cell types (Smiley *et al*., 1991). In the present experiments, no difference was noted in membrane potential between the two distinct mitochondrial populations (Figure 2B). Furthermore, the membrane potential observed in germinal vesicle, metaphase I and metaphase II oocytes obtained at oocyte retrieval was not significantly different (Figure 2C), suggesting that no major changes in metabolism occur during oocyte maturation. These data suggest that the pattern of aggregation of mitochondria within the human oocyte is independent of the mitochondrial membrane potential.

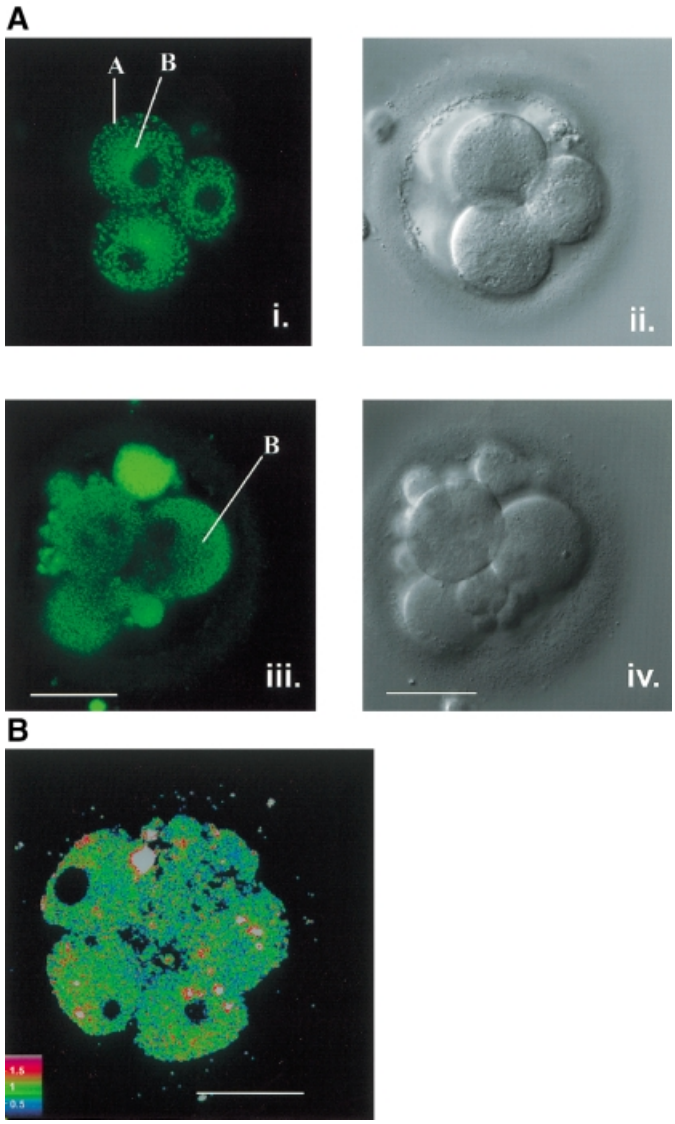
Mitochondrial aggregation patterns and membrane potential in metaphase II oocytes

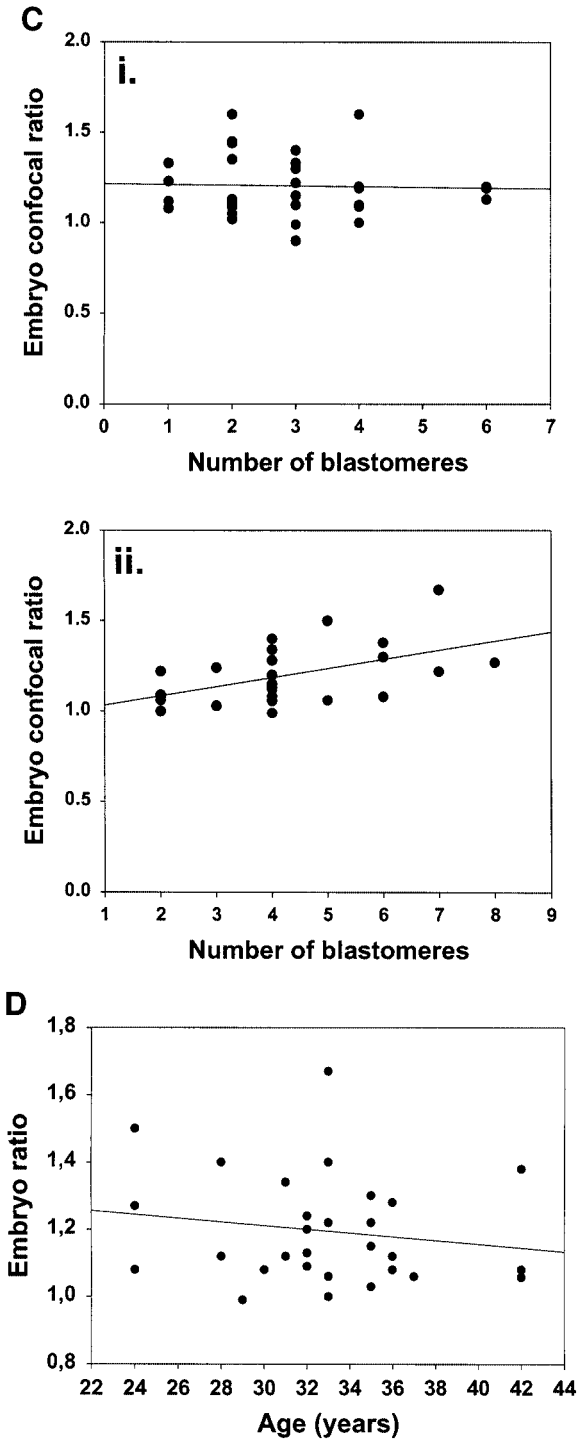
The mitochondrial aggregation pattern of fresh metaphase II human oocytes was characterized by two distinct populations, as described above. However, distinct zones of distribution of the two populations were noted. Germinal vesicle-like mitochondrial aggregation patterns (type A) were most commonly observed in proximity to the plasma membrane of fresh metaphase II oocytes. In contrast, the type B pattern was often observed both directly under the plasma membrane and towards

the centre of oocytes (Figure 3A). The localization of these two patterns suggested a degree of polarization of the oocyte, although this was not correlated with the position of the polar body (data not shown). Oocytes aged in culture were characterized by a more uniform pattern of mitochondria of type B (Figure 3A). These data suggest that the transition of mitochondria from aggregation pattern type A to type B depends on the time of culture, and hence cytoplasmic maturity. Analysis of the confocal ratio of aged oocytes revealed a significant drop in mitochondrial membrane potential ($P < 0.001$; Figure 3B). The mitochondrial aggregation pattern of fresh metaphase II oocytes was not noted to be related to the maternal age of the donor (data not shown). However, the mitochondrial membrane potential of fresh metaphase II oocytes was inversely correlated with maternal age ($r = –0.78$, $P = 1.2\times10^{-17}$, $n = 81$; Figure 3C and Table II).

Mitochondrial aggregation patterns and membrane potential in preimplantation embryos

The analysis of mitochondrial aggregation patterns in individual blastomeres of developing preimplantation embryos revealed similar trends to those observed in unfertilized





oocytes. However, the patterns were more distinct and independent. In morphologically high-grade embryos, mitochondrial pattern type A was clearly distributed at the periphery of the cytoplasm, while type B localized towards the centre of the blastomere. Furthermore, mitochondria were often found to be highly concentrated on one side of the blastomere nucleus, presumably in the region of the centrosome (Figure 4A). In complete contrast, morphologically poor quality embryos were characterized by a uniform localization of mitochondria of aggregation pattern type B (Figure 4A). The membrane potential of mitochondria in individual blastomeres from preimplantation embryos was determined by confocal microscopy. Again, no heterogeneity of mitochondrial membrane potential within the cytoplasm was observed (Figure 4B). The rate of development of preimplantation human embryos was measured by counting the number of blastomeres present at a specific time point after fertilization. Regression analysis was used to test whether this was correlated with the intrinsic mitochondrial activity of the embryo. In our analysis, a clear relationship between embryo development and mitochondrial membrane potential was observed on day 3 of culture only ($r = 0.49$, $P = 0.006$, $n = 29$, for day 3 embryos; $r = –0.02$, $P =$ not significant, $n = 34$, for day 2 embryos; Figure 4C). These data suggest that morphologically high grade preimplantation embryos are characterized by a higher mitochondrial membrane potential than slow-developing or blocked embryos, but this is apparent only when embryos are cultured to day 3 after fertilization. Replotting the data for all embryos obtained against maternal age again revealed a negative correlation ($r = –0.51$, $P = 0.00002$, $n = 63$; Figure 4D and Table III), confirming that mitochondrial activity is negatively related to maternal age in embryos as well as oocytes. Partial regression analysis was used to test whether mitochondrial activity was strongly correlated with embryo development, or whether this correlation was simply due to differences in the maternal age of the patients donating embryos. The partial regression analysis caused a slight, non-significant reduction in the correlation between embryo mitochondrial activity and embryo development (corrected $r = 0.47$, $P = 0.01$, $n = 29$; see Table IV), but did not alter the outcome of this correlation. These data suggest that embryo development is strongly correlated with embryo mitochondrial activity, and that maternal age is a further influence on this factor. No significant differences were observed when embryo mitochondrial activity was plotted against either sperm count or sperm motility (data not shown). These data demonstrate that maternal age and not sperm quality strongly influences the mitochondrial membrane potential in developing embryos, and suggest that this affects embryo quality.

Table III. Relationship between mitochondrial activity and maternal age in developing embryos

	Maternal age (years)				
	20–24	25–29	30–34	35–39	>40
No. of patients	2	3	7	7	5
No. of embryos	8	10	21	16	8
Confocal ratio[a]	1.4 ± 0.3	1.3 ± 0.2	1.2 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
95% CI	0.21	0.11	0.06	0.05	0.09

[a]Values are mean ± SD.
CI = confidence interval.

Table IV. Partial regression analysis of embryo mitochondrial activity with the rate of embryo development, correcting for maternal age

	r	*P*
Simple regression	0.49	0.006
Partial regression	0.47	0.01

Test performed at the 95% level ($n = 29$).

Discussion

In this study, ratiometric confocal microscopy was used to measure the membrane potential and hence the activity of mitochondria in human oocytes and preimplantation embryos. Relationships between mitochondrial activity and embryo quality were defined, and maternal age was shown to be a strong influence on these factors.

Maturing human oocytes are characterized by diverse cyto-

Figure 4. Mitochondrial aggregation patterns and activity in developing preimplantation embryos. (**A**) Mitochondrial aggregation patterns in good and poor quality embryos observed on day 3 after oocyte retrieval. Images on left are fluorescence images; those on right are taken from the transmitted light channel of the confocal microscope. (i., ii.): A morphologically grade 1 embryo observed on day 3 after oocyte retrieval. The mitochondrial map shows two clear aggregation patterns of mitochondria in individual blastomeres and distinct localization of the type B pattern to a region near the blastomere nucleus (see B). The image is representative of 12 similar embryos. (iii., iv.): Mitochondrial aggregation patterns in a morphologically poor quality 3-cell grade 2 embryo observed on day 3 after oocyte retrieval. Blastomeres are characterized by type B mitochondria without any observable localization. The images are representative of 11 examples. Scale bar = 50 μm. (**B**) Ratio of mitochondrial activity in developing embryos. Images from the red confocal channel were divided by images from the green channel. The result is a map of mitochondrial activity. No heterogeneity is observed. These images are representative of 63 embryos analysed. The colour bar shows the ratio. Areas of white colour are embryo fragments. Scale bar = 50 μm. (**C**) Relationship between embryo mitochondrial activity and cleavage rate. These are scatter graphs of the embryo confocal ratio against embryo quality scored as number of blastomeres present either on day 2 of culture or day 3. (i.): day 2; the graph is compiled using a total of 34 embryos not utilized for transfer on day 2 in a total of 11 patients. The regression line shows a non-significant correlation coefficient of –0.03 and $P = 0.9$. (ii.): day 3; the graph is compiled using a total of 29 embryos not utilized for transfer on day 3 in a total of 13 patients. The regression line shows a highly significant correlation coefficient of 0.49 and $P = 0.007$. (**D**) Relationship between embryo mitochondrial activity and maternal age. The same readings recorded in (**C**) were plotted against maternal age. The regression line shows a correlation coefficient of –0.27 and $P = 0.005$.

plasmic morphological characteristics visible under the light microscope. The data presented here suggest that the aggregation pattern of mitochondria corresponds closely to the gross oocyte morphology. Two major mitochondrial aggregation patterns were visualized. The first pattern (type A) was more common in immature, germinal vesicle-stage oocytes. Oocyte maturation proceeded with the appearance of a second pattern (type B). Of particular interest was the observation that type B was formed mainly towards the centre of the oocyte, whereas type A was confined to the subplasma membrane region. In fact, the two patterns often showed a polarized distribution. These data suggest that mature human oocytes are polarized, at least with respect to mitochondrial aggregation. In this study, the relationship between the mitochondrial aggregation pattern and the position of the meiotic apparatus was not examined, but one hypothesis would be that the polarization of the mitochondrial morphology is an indicator of the animal-vegetal pole of the oocyte. This is particularly interesting because the polarization of mitochondrial aggregation did not correspond with the position of the polar body, which is already known to be an unreliable indicator of the position of the meiotic apparatus (Garello *et al*., 1999; Van der Westerlaken *et al*., 1999). It is not known which cytoplasmic determinant determines mitochondrial aggregation: however, the data presented here suggest that cytoplasmic maturity may play a role. Despite the morphological polarization of oocytes, the data did not reveal any localized heterogeneity of mitochondrial activity, suggesting that the two effects are unrelated. Human preimplantation embryos were also characterized by two distinct populations of mitochondria. Preimplantation embryos were further characterized by a concentration of mitochondria to one side of the nucleus. In the present data, no heterogeneity in mitochondrial activity was observed, although this has been reported previously (Bavister and Squirrel, 2000). These data indicate that the type of mitochondrial aggregation observed is independent of activity in human oocytes, and suggest that factors localized within the oocyte and embryo cytoplasm determine these patterns.

The pattern of mitochondrial aggregation in oocytes was not found in the present investigation to be correlated with localized regions of mitochondrial activity. However, mitochondrial activity did show distinct relationships to diverse factors in oocytes and embryos. No changes in mitochondrial activity were noted during oocyte maturation. However, it must be noted that these data were obtained using immature oocytes obtained after oocyte retrieval and therefore 36 h after the administration of HCG. The data cannot therefore exclude increases in oocyte metabolism as a direct effect of HCG administration. Fresh oocytes obtained at oocyte retrieval were characterized by a mitochondrial activity that showed a negative correlation with maternal age. Furthermore, the mitochondrial activity in blastomeres of preimplantation embryos also showed a strong negative correlation with maternal age. Mitochondrial activity was positively correlated with the rate of development of human embryos, but only when these embryos were analysed on day 3 after oocyte retrieval. A reasonable explanation for this seemingly odd observation is that day 2 embryos have not always fully entered the cleavage stage of embryo development, and therefore the number of blastomeres present is not indicative of the rate of development. No correlation was observed with the quality of spermatozoa used to inseminate the partner's oocytes. This is perhaps not surprising when it is considered that the sperm mitochondria play no part in the development of the embryo (Hecht *et al*., 1984); however, the data further demonstrate that the spermatozoa do not introduce factors into the oocyte cytoplasm that directly affect the level of respiration.

Taken together, these data suggest that an accumulation of factors in the ovaries of older women leads to a reduced efficiency of mitochondrial respiration, with a subsequent negative effect on embryo development. It is not known whether these factors are genetic; for example, the accumulation of mutations in mitochondrial DNA (Keefe *et al*., 1995; but see Brenner *et al*., 1998; Barritt *et al*., 1999; Perez *et al*., 2000; Steuerwald *et al*., 2000), or environmental, for example in the accumulation of reactive oxygen species-induced damage to oocytes within the ovary (Wallace, 1992; Shigenaga *et al*., 1994). However, the present data underlie the previously reported decrease in oocyte quality and consequent increase in aneuploidy in embryos from older patients (Gaulden, 1992; Munne *et al*., 1995; Dailey *et al*., 1996; Janny and Ménézo, 1996) and hence suggest reasons for the lower embryo implantation rate in these couples. The data suggest that the transfer of cytoplasm from young donors to older recipient oocytes (Cohen *et al*., 1997, 1998) increases embryo quality by introducing mitochondria with a higher activity than that of the recipient (Brenner *et al*., 2000). The transfer of donor mitochondria may improve the recipient embryo quality during the early stages of embryo development, thus increasing the possibilities for the embryo to implant.

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