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Low oocyte mitochondrial DNA content in ovarian insufficiency

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BACKGROUND: Mitochondrial biogenesis and bioenergetics play an important role in oocyte maturation and embryo development. We have investigated the relationship between defective mitochondrial biogenesis and the lack of oocyte maturity observed during IVF procedures with patients suffering from ovarian dystrophy and ovarian insufficiency. METHODS: We used real-time quantitative PCR to quantify mitochondrial DNA (mtDNA) in 116 oocytes obtained from 47 women undergoing the ICSI procedure. We compared the mtDNA content of oocytes from women with a normal ovarian profile with that of oocytes from women with ovarian dystrophy and ovarian insufficiency. RESULTS: We found an average of 256 000 ± 213 000 mitochondrial genomes per cell. The mean mtDNA copy number was not significantly different in ovarian dystrophy compared with controls, but it was significantly lower in oocytes from women with ovarian insufficiency (100 000 ± 99 000, $P < 0.0001$). CONCLUSIONS: Our results suggest that low mtDNA content is associated with the impaired oocyte quality observed in ovarian insufficiency.

Key words: IVF/mitochondrial DNA/oocyte quality/ovarian insufficiency

Introduction

Although assisted reproductive technology has greatly progressed over the past two decades, the average rate of pregnancy obtained with IVF by embryo transfer is still low at 25% (FIVNAT, 2003), and even lower, perhaps no more than 10%, when calculated for each embryo transferred. The low efficacy of the method may be due to poor oocyte quality. The principle that embryogenesis is rooted in oogenesis has been admitted for nearly a century. During folliculogenesis, the oocyte gradually acquires the ability to mature, to be fertilized and finally to develop into a viable embryo (Gosden, 2002). Starting at the primordial follicular stage, the oocyte takes months to complete its growth phase, increasing its total volume >100-fold. This growth involves qualitative and quantitative changes in key molecules for metabolism, structure and information (Bell *et al.*, 1997; Ji *et al.*, 1997; Gandolfi and Gandolfi, 2001; Gosden, 2002).

During follicular growth, the number of oocyte mitochondria rises from ~10 000 to ~200 000 (Jansen and de Boer 1998). The functional status of the mitochondria contributes to the quality of oocytes and probably plays an important role in the process of fertilization and embryo development (Cummins, 2004). In particular, the developmental potential of the embryo and the outcome of IVF have been shown to be related to the ATP content of human oocytes (Van Blerkom *et al.*, 1995). In addition to their role in energetic conversion, mitochondria participate in numerous other essential cell functions such as the regulation of apoptosis, calcium homeostasis, Fe–S protein synthesis, and pyrimidine and haem synthesis (Delbart, 2000). In an earlier report, we found a significant reduction in mitochondrial DNA (mtDNA) content in cohorts of oocytes examined after failure of an IVF procedure (Reynier *et al.*, 2001). We hypothesized that a defect in mitochondrial biogenesis together with insufficient mitochondrial mass may have led to defective oocyte maturation and hence to the poor outcome of IVF.

Mitochondria are frequently organized in dynamic interconnected networks, making the precise count of mitochondria technically difficult (Rube and van der Bliek, 2004). However, the quantification of mtDNA by means of real-time PCR provides a good evaluation of the mitochondrial mass in single cells, such as oocytes, because of the sensitivity of the method and the high correlation between the quantity of mtDNA and the mitochondrial mass (Wu *et al.*, 2002).

In order to investigate the relationship between oocyte quality and the mitochondrial mass, we determined the mtDNA content of oocytes in the context of the two main ovarian disorders, ovarian dystrophy and ovarian deficiency, as compared with the normal ovarian profile. We used real-time quantitative PCR to determine the mtDNA content of 116 oocytes obtained from 47 women undergoing IVF.

All these oocytes had been discarded during ICSI procedures since they lacked the first polar body.

Materials and methods

Groups of patients

The oocytes were classified into three groups according to the ovarian profile of the patients (Hazout, 1999). We excluded women presenting with mixed ovarian disorders, such as multifollicular ovaries with signs of ovarian insufficiency.

Group 1 consisted of 39 oocytes collected from 14 patients presenting a normal ovarian profile defined on day 3 of a spontaneous cycle by hormonal assay interms of FSH <5 IU/l, LH <6 IU/l, estradiol <45 pg/ml and inhibin B >45 pg/ml; and by ovarian ultrasonography interms of >3 antral follicles per ovary.

Group 2 consisted of 47 oocytes collected from 16 patients presenting a profile of ovarian dystrophy defined on day 3 of a spontaneous cycle by hormonal assay as an FSH/LH ratio <1 with FSH <5 IU/l; and/or by ovarian ultrasonography as higher than the normal number (>10) of small follicles (2–8 mm in diameter) encircling the ovarian cortex like a string of pearls, with or without the increase of stroma. All patients except one presented normal androgen levels. In this group, 10 patients had an FSH/LH ratio <1 with a normal FSH value. Six of these 10 patients had an increased number of peripheral antral follicles. The other six patients had a normal FSH/LH ratio but all showed signs of ovarian dystrophy on ultrasonographic examination.

Group 3 consisted of 30 oocytes collected from 17 patients presenting a profile of ovarian insufficiency defined on day 3 of a spontaneous cycle by hormonal assay: FSH >8 IU/l (according to our laboratory norms) and/or by ovarian ultrasonography: <4 antral follicles per ovary. Fifteen of the 17 patients presented high basal FSH levels (>8 IU/l) on day 3. Among them, seven presented a low antral follicle count (<4 follicles per ovary). The two remaining patients presented normal FSH levels but elevated estradiol levels and low antral follicle counts.

As expected, the mean age of women was significantly higher in the group with ovarian insufficiency, group 3 (35.4 ± 4.1 years), than in the group with a normal ovarian profile, group 1 (31.0 ± 2.4 years) (Mann–Whitney: $P = 0.001$). There was no significant age difference between the normal ovarian profile group and the multifollicular ovarian profile group, group 2 (30.3 ± 3.2 years).

The average number of recombinant FSH IU used for the stimulation was significantly different for the three groups: 14×75 IU for the group with ovarian dystrophy, 22×75 IU for the group with the normal ovarian profile, and 39×75 IU for the group with ovarian insufficiency (Kruskall–Wallis: $P < 0.0001$).

The estradiol peak level was not statistically different between the three groups.

The number of oocytes retrieved was lower in the ovarian insufficiency group than in the other two groups (Kruskall–Wallis: $P = 0.03$).

Oocyte samples

The ethics committee of the University Hospital of Angers approved the plan of our study on oocytes discarded during ICSI procedures. Follicular growth was stimulated by recombinant FSH associated with a GnRH agonist. Ovulation was induced with HCG and the oocytes were retrieved by means of a transvaginal probe. Cumulus cells were then removed from each oocyte using gentle pipetting with hyaluronidase (80 IU Fertipro, Beernem, Belgium). For the ICSI procedure, only oocytes presenting their first polar body can be injected. Oocytes which had not extruded their first polar body 4 h after removal of the follicular cells [blocked at prophase I (germinal vesicle) or blocked after the prophase I state] were retained for our study. A total of 116 isolated oocytes (69 in prophase I, and 47 blocked between prophase I and metaphase II) were collected individually from 47 women over a 12 month period. The indication for ICSI had been male infertility in 42 couples (i.e. the presence of $<100\,000$ motile spermatozoa available after sperm preparation) and previous fertilization failure in five couples. Each oocyte was placed in 50 µl of IVF-30 medium (IVF Scandinavian, Stockholm, Sweden). The oocytes were stored at −20°C until DNA extraction, performed within a month following collection.

Preparation of DNA

DNA was extracted from each oocyte by means of the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's recommendations. The DNA was bound specifically to glass fibres following the combined action of a chaotropic agent (guanidine), a detergent (Triton X-100) and the enzyme proteinase K. After washing, the silica-bound DNA was eluted with 200 µl of pre-warmed (72°C) elution buffer and maintained at 4°C. The extraction efficiency, assessed as described elsewhere (Reynier *et al.*, 2001), was >90%.

Mitochondrial DNA quantification by real-time PCR

We used a Roche LightCycler to determine the mtDNA copy number using the LightCycler-Faststart DNA master SYBR Green 1 kit (Roche, Mannheim, Germany) as described elsewhere (Reynier *et al.*, 2001). Briefly, 20 µl PCR mixtures were prepared as follows: 1× buffer containing 4 mmol/l $MgCl_2$, 0.2 mmol/l dNTPs, 0.5 mmol/l of both primers (D41 and R56), SYBR Green I dye, 0.25 U of hot-start *Taq* DNA polymerase and 10 µl of the extracted DNA or 10 µl of standard with a known copy number. The reactions were performed as follows: initial denaturing at 95°C for 7 min and 40 cycles at 95°C for 1 s, 58°C for 5 s and 72°C for 13 s. The SYBR Green fluorescence was read at the end of each extension step (72°C). A melting curve (loss of fluorescence at a given temperature between 66 and 94°C) was analysed in order to check the specificity of the PCR product. For each run, a standard curve (log of the initial template copy number on the abscissae, and the cycle number at the crossing point on the ordinates) was generated by using five 10-fold serial dilutions (100–1 000 000 copies) of the external standard. This curve allowed the determination of the starting copy number of mtDNA in each sample. All samples were tested twice. The raw data were then multiplied by 20 to calculate the total mtDNA content in each oocyte. The precision of the real-time PCR mtDNA quantification was assessed as described elsewhere (Reynier *et al.*, 2001). The intra- and inter-assay coefficients of variation ranged from 3.9 to 9.1% and from 9.3 to 12.7%, respectively.

Statistical analysis

Since the distribution of the variables analysed was non-Gaussian, all comparisons were made using the non-parametric Mann–Whitney and Kruskal–Wallis U-tests. Differences were considered significant at $P < 0.05$. Statistical analysis was performed with SPSS software, version 10.1 (SPSS, Chicago, IL)

Results

The average mtDNA copy number of the 116 oocytes was 256 000 ± 213 000 with a high inter-oocyte variation (11 000–903 000). There was no significant difference in

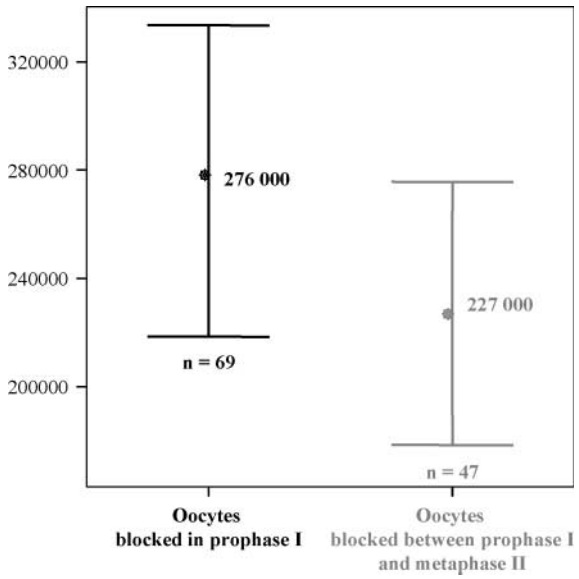

Figure 1. Comparison of mtDNA content in oocytes blocked at prophase I ($n = 69$) and those blocked at metaphase I ($n = 47$). No significant differences were found. The results are presented as variation diagrams: the points represent the average values and the bars indicate the 95% confidence intervals of the means.

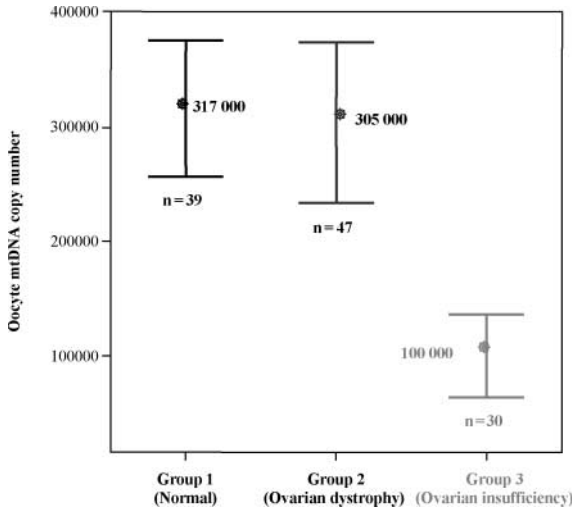

Figure 2. Comparison of mtDNA content in oocytes from the three groups of patients: Group 1 (normal ovarian profile), group 2 (ovarian dystrophy) and group 3 (ovarian insufficiency). There were significantly fewer mtDNA copy numbers in group 3 than in the other two groups ($P < 0.0001$).

the mtDNA copy number of oocytes blocked in prophase I (276 000 ± 238 000) and those blocked between prophase I and metaphase II (227 000 ± 166 000) (Mann–Whitney: $P = 0.5$) (Figure 1). Moreover, there was no significant difference between the oocytes analysed in the present study and the unfertilized oocytes in metaphase II described in our previous report (Reynier *et al.*, 2001). This finding implies that the number of mitochondria is fixed at the time of ovulation and that terminal nuclear oocyte maturation (germinal vesicle breakdown and extrusion of the first polar body) is independent of the mtDNA copy number. We may therefore compare the three groups of oocytes studied.

There was no significant difference in the average mtDNA copy number between the oocytes of group 1 with the normal ovarian profile (317 000 ± 184 000) and those of group 2 with the ovarian dystrophy profile (305 000 ± 240 000). In contrast, the average mtDNA copy number of group 3 with the ovarian insufficiency profile (100 000 ± 99 000) was significantly lower than that of group 1 ($P < 0.0001$) and group 2 ($P < 0.0001$) (Figure 2).

Discussion

Mitochondria contain their own specific genome, a circular double-stranded DNA molecule containing 16.6 kb encoding 13 essential subunits of the respiratory chain complexes that provide the main ATP supply of the cell (Anderson *et al.*, 1981). Most mammalian cells contain between several hundred and several thousand mtDNA molecules, the oocytes being the richest cell of the body in terms of mtDNA content. The average mtDNA copy number we found, i.e. 256 000 ± 213 000, is comparable with previous quantitative reports on unfertilized human oocytes that have reported average mtDNA copy numbers of 138 000 (Chen *et al.*, 1995), 193 000 (Reynier *et al.*, 2001) and 314 000 (Steuerwald *et al.*, 2000). The disparity between absolute values between studies probably reflects the great inter-oocyte variation observed in mtDNA copy numbers (range 50 000–1 550 000).

In the present study we have attempted to examine the relationship between oocyte mtDNA content and female infertility. To determine the best protocol for ovarian stimulation and oocyte collection, candidates for IVF are classified according to the results of a set of clinical, biological and ultrasound investigations (Hazout, 1999). The three main categories correspond to women with a normal ovarian profile, an ovarian dystrophy profile and an ovarian insufficiency profile. The precise pathophysiological mechanisms responsible for ovarian dystrophy and ovarian insufficiency are unknown. However, the quality, the fertilization and the cleavage rates of oocytes are impaired in patients with ovarian dystrophy (Dor *et al.*, 1990; Aboulghar *et al.*, 1997; Engmann *et al.*, 1999; Plachot *et al.*, 2003). Similarly, ovarian insufficiency is associated with cycle cancellation, poor oocyte quality and decline of IVF success rates (Pellicer *et al.*, 1987; Jenkins *et al.*, 1991) resulting from diminished ovarian reserve at the qualitative and quantitative levels (Nasseri *et al.*, 1999; Bancsi *et al.*, 2002).

We found two distinct ranges of mtDNA levels with no overlap between patients with ovarian insufficiency and those with a normal profile. Thus, ovarian insufficiency is clearly associated with specifically low mtDNA content, probably indicating abnormal mitochondrial biogenesis during oocyte growth and reflecting cytoplasmic immaturity. The range of oocyte mtDNA content in women with ovarian dystrophy, in contrast to those with ovarian insufficiency, was identical to that of women with a normal ovarian profile. It is thus likely that the poor oocyte quality associated with ovarian insufficiency and ovarian dystrophy has different molecular

aetiologies. Our results suggest that in the case of ovarian dystrophy, impaired oocyte maturity is unrelated to mitochondrial biogenesis. This is corroborated by the fact that the biological effects of oocyte immaturity in ovarian dystrophy and ovarian insufficiency are quite different. During IVF procedures, the proportion of immature oocytes is significantly higher and the rate of fertilization is significantly lower in patients with ovarian dystrophy than in patients with a normal ovarian profile. Thus, although there exists a general immaturity in the cohort of oocytes in ovarian dystrophy, once fertilized, a subset of mature oocytes develop into good quality embryos (Dor *et al.*, 1990; Hardy *et al.*, 1995; Engmann *et al.*, 1999). This is very different from the case of ovarian insufficiency, which is characterized by poor oocyte quality as well as poor embryo quality.

Our results raise the question of the functional consequences of impaired mitochondrial biogenesis observed in the oocytes of patients with ovarian insufficiency. In the embryo, the generation of ATP during the pre-compaction stages is largely dependent upon oxidative phosphorylation (Bavister and Squirrell, 2000; Thompson *et al.*, 2000; Van Blerkom *et al.*, 1998, 2000). The variation of the ATP content of human oocytes has been associated with the developmental competence of embryos (Van Blerkom *et al.*, 1995; Wilding *et al.*, 2001). Indeed, ATP seems to be generated prior to cavitation by pre-existing oocyte mitochondrial proteins and transcripts that must be produced under the control of the maternal nuclear and mitochondrial genome during oogenesis (Cummins, 2004). The low mitochondrial mass may be insufficient to constitute the necessary energetic reserves during follicular growth. Moreover, mitochondria are involved in many essential cellular processes other than ATP production, so that several functions, such as regulation of apoptosis, calcium homeostasis, pyrimidine and haem synthesis and other metabolic pathways, could be impaired by the low mitochondrial mass and lead to the poor outcome of IVF.

The importance of mitochondria in oocyte quality and embryo development is highlighted by the cytoplasmic transfer procedure. In animal experiments, normal developmental potential has been restored to eggs with ooplasmic deficiencies by the transfer of ooplasm from normal eggs (Flood *et al.*, 1990; Levron *et al.*, 1996). In humans, inter-oocyte cytoplasmic transfer has been used in an attempt to overcome pregnancy failure due to poor oocyte quality (Cohen *et al.*, 1997). A relationship between mitochondrial function and developmental capacity may explain the promotion of the developmental potential of seemingly incompetent oocytes by the introduction of ooplasm aspirated from a normal oocyte. Although the precise cellular structures and macromolecules thus introduced into the oocyte are unknown, mitochondria could represent an important component. Indeed, the isolation and transfer of mitochondria between oocytes is known to increase ATP production in the recipients, with the persistence of activity in the transferred mitochondria (Van Blerkom *et al.*, 1998). Moreover, the microinjection of small numbers of mitochondria into mouse oocytes prevents the onset of apoptosis (Perez *et al.*, 2000).

In conclusion, we found that the mtDNA content was particularly low in all the oocytes retrieved from patients with ovarian insufficiency, whereas oocytes from patients with ovarian dystrophy contained normal quantity of mitochondrial genomes. We believe that ovarian insufficiency and the associated poor oocyte quality are specifically linked with impaired mitochondrial biogenesis, probably related to disorders in cytoplasmic maturation. Further research into the pathophysiological mechanisms involved should allow us to establish whether impaired mitochondrial biogenesis is the central abnormality responsible for poor oocyte quality or simply evidence of impaired oogenesis.

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