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Mitochondrial DNA variations in ova and blastocyst: Implications in assisted reproduction

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ABSTRACT

Mitochondrial DNA (mtDNA) of oocyte is critical for its function, embryo quality and development. Analysis of complete mtDNA of 49 oocytes and 18 blastocysts from 67 females opting for IVF revealed 437 nucleotide variations. 40.29% samples had either disease associated or non-synonymous novel or pathogenic mutation in evolutionarily conserved regions. Samples with disease associated mtDNA mutations had low fertilization rate and poor embryo quality, however no difference in implantation or clinical pregnancy rate was observed. Screening mtDNA from oocyte/blastocyst is a simple, clinically reliable method for diagnostic evaluation of female infertility and may reduce risk of mtDNA disease transmission.

1. Introduction

Infertility is a multi-factorial disorder and common etiologies attributed to female infertility are the defects of the gametogenesis, gamete function, ovulatory defects, fertilization failure, implantation defects and embryo loss. The molecular bases of these abnormalities are not completely understood. Development of a functionally competent oocyte with the potential to produce healthy offspring is a complex and energy dependent process in which mitochondrial genome plays a critical role. The expression of mitochondrial protein and the copy number of mitochondrial DNA (mtDNA) vary at different stages of oogenesis and embryonic development (Fig. S1).

The transition of primordial follicle to a mature oocyte is accompanied by increase in mtDNA copy number (Jansen and de Boer, 1998; St John et al., 2010). A mature human oocyte (MII stage) may contain 50,000 to 1,550,000 copies of mtDNA (Barritt et al., 2002; May-Panloup et al., 2005a; Reynier et al., 2001), which accounts for about one third of the total DNA content of an oocyte (Piko and Matsumoto, 1976). Post-fertilization embryo expresses either very low level of mtDNA replication factors or there is no expression, indicating that the cell division of the early embryo proceeds without mtDNA replication (May-Panloup et al., 2005a, 2005b; Spikings et al., 2007). Till the implantation of the embryo in the uterus, the number of mtDNA copies per blastomere progressively decreases with each embryonic cell division (Leese and Barton, 1984; St John et al., 2010). Thus, by the time inner cell mass are set-aside in the blastocyst, the mtDNA copy number is reduced to ~1000 per cell. Therefore, the decrease in mtDNA copy number gradually reduces the capacity to generate adenosine triphosphate (ATP) through oxidative phosphorylation (OXPHOS) (Leese, 1995; Van Blerkom et al., 2002).

Lack of redundant copies of the mtDNA may render the oocyte susceptible to mutations, which in turn may lead to energy crisis due to dysfunction of OXPHOS. Any quantitative or qualitative defect in the mitochondrial genomic constituent may lead to negative impact on the bio-energetic competence of oocyte, which may ultimately result in infertility. Further, the exclusive maternal inheritance without the mitochondrial supplement from the fertilizing sperm makes the developing embryo solely dependent on the mitochondrial pool of the oocyte (Sutovsky et al., 1999).

Direct correlations between the mtDNA content of oocyte and age of the donor suggest reproductive senescence of the oocyte with age (Chan et al., 2005). Barritt et al. (1999) analyzed the mtDNA from 5835 bp to 16065 bp and detected 23 different rearrangements with no age-related association in human oocyte (Barritt et al., 1999). A study by Jacobs et al. (2007) revealed that hetroplasmic mutations in

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oocytes could be a potential cause in the development of OXPHOS diseases (Jacobs et al., 2007).

Most of the genetic studies have been performed using the DNA isolated from blood. However, germline and early embryo (blastocyst) might provide a better insight to our understanding of the germline or tissue-specific genetic abnormalities. Studies on the oocyte/blastocyst are limited due to the logistics and ethics involved in obtaining the oocyte and the early embryo. Technical limitations of genome analysis at the single cell level, also add to the constraints. To the best of our knowledge, so far, no study has analyzed the whole mitochondrial genome of oocyte/blastocyst to assess the impact of mtDNA variations with fertilization rate, embryo quality, implantation rate and Assisted Reproduction Technique (ART) outcome. Hence, we have analyzed the complete mitochondrial genome of oocyte and blastocyst of females opting for ART in order to assess the implications of mtDNA variations on clinical parameters, indices of embryo development, and ART outcome.

2. Material and methods

2.1. Subjects

A total of 49 unfertilized oocytes and 18 blastocysts were retrieved from 67 females opting for assisted reproduction in the ART centre, Department of Obstetrics and Gynaecology, All India Institute of Medical Sciences (AIIMS), New Delhi; and IVF centre, Army Research and Referral Centre, New Delhi. The couples had normal chromosomal complement and hormone profile and the male partners had no Yq micro-deletions in the *AZF* region and showed normal sperm parameters.

Relevant clinical details, medical history and pedigree were documented on a predesigned proforma. The study was approved and conducted in accordance with ethical guidelines. An informed written consent was obtained from all the subjects, who have participated in this study.

2.2. Oocyte retrieval

Ovulation induction was performed using the standard protocol followed at ART centre, AIIMS and IVF centre, Army Research and Referral Centre. The choice of stimulation protocol was between long protocol with down-regulation using the Gonadotrophin Releasing Hormone (GnRH) agonist lupride (German remedies, India) or a GnRH antagonist protocol using multi dose cetrorelix acetate (Merck Serono, USA). The gonadotrophin used was either urinary or recombinant, the dose of which varied from patient to patient based on their age, follicle stimulating hormone (FSH) and anti-mullerian hormone (AMH) levels and body mass index (BMI). Oocyte retrieval was done by transvaginal ultrasound-guided aspiration, 35–36 h after the administration of human chorionic gonadotrophin (HCG) (Sun Pharma, India). The cumulus–oocyte complexes were isolated into Flushing Media (Medicult, Denmark). Conventional IVF sperm insemination was performed after density centrifugation 38 h after giving HCG. Oocytes were assessed for fertilization 16–18 h after insemination. Embryo cleavage and quality were evaluated on day 2, prior to transfer.

2.3. Embryo classification

Embryo morphology was scored from grade I to IV according to the shape of the blastomeres and the amount of detached anuclear fragments (Fig. S2). Embryos with equal-sized cells and no fragmentation were categorized as grade I; embryos with <20% fragmentation with equal or unequal-sized cells were categorized as grade II; embryos with 20–50% fragmentation with equal or unequal-sized cells were categorized as grade III; and the embryos with >50% fragmentation were categorized as grade IV.

Embyro transfer (ET) was done on day 2 or 3 or 5 under ultrasound guidance using Cook's soft catheter. Luteal support was given with progesterone 100 mg daily intramuscular injections. Pregnancy was checked 14 days after ET with serum βhCG levels and clinical pregnancy was confirmed with a viable fetal heart on a trans-vaginal sonography, 6 weeks after ET.

2.4. Sample preparation

Unfertilized metaphase II (MII) oocyte, which showed no evidence of fertilization (i.e., failure to demonstrate a second polar body and two pronuclei) and compromised grade IV embryos that were arrested or abnormal and not suitable for implantation or cryopreservation were considered for the study. To eliminate the possibility of contamination by sperm mtDNA, the zona pellucida of all oocyte were removed using acidified Tyrode's solution. The cells were then washed with PBS in plastic dishes and then transferred to labeled 0.2 ml eppendorf tubes with 1–2 μl of IVF media (Vitrolife, Sweden) and stored at −20 °C till further processing.

2.5. Cell lysis and DNA extraction

The oocyte were thawed at room temperature and then incubated in 10 μl lysis buffer (50 mM Tris–HCl, pH 8.5, 0.1 mM EDTA, 0.5% Tween-20, 200 μg/ml proteinase K) at 55 °C for 2 h to lyse the cell. Finally, proteinase K was heat inactivated at 95 °C for 10 min. The cell lysate was subsequently used for long PCR to amplify the complete mtDNA into two large fragments (Fig. S3).

2.6. PCR amplification and sequencing

Prior to sequencing, mtDNA was amplified from a single cell (present in cell lysate), using a two-step approach. In the first step, entire mtDNA was amplified as two large overlapping amplicons of 7.8 kb and 9.3 kb, using two sets of following primers:

(1F: tcatttttattgccacaactaacctcctcggactc, 1R: cgtgatgtcttatttaaggggaacgtgtgggctat; 2F: acatagcacattacagtcaaatcccttctcgtccc, 2R: attgctagggtggcgcttccaattaggtgc).

Long range amplification of entire mtDNA was done by a multiplex PCR in 30 μl PCR reaction using 10 μl of the cell lysate and 20 μl of PCR constituents, including 5 pM of each primer (Sigma chemicals, India), 2.5 U Taq Polymerase, 2 μl of dNTPs (2.5 mM each), 2 μl 10× PCR buffer and 2 μl 25 mM $MgCl_2$ (Takara Bio Inc., Japan). The initial denaturation step was at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing-extension at 68 °C for 9 min and final extension at 68 °C for 15 min. The amplicons were then diluted 40 folds, which served as a template for the second round of PCR.

In the second round of PCR, the diluted amplicon was re-amplified by nested PCR, using 24 sets of primers to generate overlapping fragments as described previously (Rieder et al., 1998; Vanniarajan et al., 2006). Precautions were taken to prevent contamination and a buffer-only negative control and genomic DNA, isolated from blood, was used as a positive control in all PCR reactions. Amplicons were electrophoresed using 2% agarose gel. The cycle sequencing reaction was carried out using 1 μl of amplicon from the nested PCR in a GeneAmp 9700 thermal cycler using BigDye Terminator ready reaction kit (Applied Biosystems, Foster City, USA) (Thangaraj et al., 2003a, 2003b). Extended products were precipitated with sodium acetate and ethanol, dried and dissolved in Hi–Di formamide, followed by analysis in an ABI 3730 automated DNA analyzer (Applied Biosystems, Foster City, USA).