

SPERM MITOCHONDRIAL DNA COPY NUMBER IS NOT A PREDICTOR OF IN VITRO FERTILIZATION/ INTRACYTOPLASMIC SPERM INJECTION (IVF/ICSI) CYCLE OUTCOMES

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STUDY QUESTION: Is sperm mitochondrial DNA copy number (mtDNA CN) associated with fertilization, usable blastocyst development, blastocyst euploidy, and live birth rates in an infertile population?

SUMMARY ANSWER: Sperm mtDNA CN is not associated with fertilization, usable blastocyst development, euploidy and live birth rates in an infertile population undergoing IVF/ICSI.

WHAT IS KNOWN ALREADY: Approximately half of infertile couples have a male factor component, however, few prognostic tests of sperm quality or function exist. Several investigations have correlated sperm mtDNA copy number with various semen parameters in both fertile and infertile men, but the

presence of possible associations between sperm mtDNA CN values and rates of usable blastocysts, euploidy, and live birth has not been assessed previously.

STUDY DESIGN, SIZE, DURATION: This is a cohort study conducted on stored sperm samples collected prospectively and used to create blastocysts transferred in a couple's first IVF cycle between 2007 and 2013 at a single large infertility center. Patients consenting to the use of otherwise discarded, whole sperm material for research purposes were evaluated for inclusion. Samples from IVF cycles utilizing surgical or cryopreserved sperm, conventional insemination, or day 3 embryo biopsy were excluded. Cycles were also excluded if the female patient had a BMI > 35 or failure of endometrial proliferation ≥ 6 mm. The primary outcome was live birth rate. Secondary outcomes included fertilization, usable blastocyst development and blastocyst euploidy rates. A sample size of 2,000 patients was chosen to achieve > 80% power to detect a 10% difference in live birth rates, which was felt to be clinically significant.

PARTICIPANTS/ MATERIALS, SETTING, METHODS: A total of 2,062 unique sperm samples used to create transferred embryos were identified. Mitochondrial DNA CN was evaluated using TaqMan® qPCR assays normalized to a nuclear control for relative quantitation. Linear regression was used to assess the relationship between relative sperm mtDNA value, total motile sperm count, paternal age and days of abstinence. Mixed effects logistic regression models were used to explore relationships between sperm mtDNA CN and fertilization, usable blastocyst development, euploidy and live birth rates.

MAIN RESULTS AND THE ROLE OF CHANCE: Lower relative sperm mtDNA content was associated with increased pre-wash sperm motility ($p < 0.001$). No significant association was identified between sperm mtDNA CN and fertilization ($p = 0.40$), usable blastocyst development ($p = 0.36$), blastocyst euploidy ($p = 0.10$), and live birth rates ($p = 0.42$) while adjusting for sperm pre-wash motility and maternal age.

LIMITATIONS, REASONS FOR CAUTION: Limitations of this study include the assumption that quantification of the mean mtDNA content of a population of sperm is representative of the individual sperm inseminated. Furthermore, it is unknown whether sperm mtDNA CN is consistent between samples collected from the same patient and, therefore, a reproducible test.

WIDER IMPLICATIONS OF THE FINDINGS: Sperm mtDNA CN was not found to be prognostic of IVF/ICSI cycles in an infertile population and, therefore, does not add to clinical care as a diagnostic test.

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Key words: SPERM MITOCHONDRIAL DNA COPY NUMBER, IVF/ ICSI OUTCOMES, SPERM QUALITY, INFERTILE POPULATION

Introduction

Male factor accounts for approximately 20% of infertility diagnoses and is implicated in up to half of cases of infertility (Thonneau *et al*, 1991). The WHO 2010 semen parameter reference values are heavily relied upon for the diagnosis of male infertility; however, these parameters do not reliably prognosticate sperm quality or reproductive function (Esteves *et al*, 2012). Many methods have been introduced in an attempt to improve the assessment of sperm quality, including DNA integrity testing, assessment of kinetic patterns, organelle morphology screening, hyaluronic acid binding, reactive oxygen species levels and total antioxidant capacity, proteomic analysis of seminal plasma and spermatozoa, and transcriptomic profiling (reviewed in Agarwal *et al*, 2015). However, no test has yet proven to definitively improve assessment of sperm quality or function in a manner that benefits patient outcomes. Therefore, in the diagnostic evaluation of the male in a couple with infertility, a test with a favorable predictive value for the reproductive function of sperm is needed.

The assessment of mtDNA CN within gametes has been an area of active research for many years. In most species, including humans, mtDNA is solely inherited from the mother (Hutchinson *et al*, 1974; Giles *et al*, 1980). Oogenesis is associated with a strong amplification of mtDNA copy number, while spermatogenesis is associated with a drastic reduction in mtDNA content (St John *et al*, 2010). This drastic reduction occurs through both the down-regulation of mtDNA transcription factors in the sperm and ubiquitin-mediated degradation of the remaining sperm mitochondria after fertilization (Cummins *et*

al, 1998; Hajjar *et al*, 2014). These processes facilitate the elimination of paternally-derived mtDNA during early embryonic development. As such, it has been shown that mature gametes with the greatest capability to achieve fertilization are oocytes with high mtDNA content, and sperm with very low mtDNA content (Reynier *et al*, 2001; Boucret *et al*, 2015; Tian *et al*, 2014), making mtDNA CN a plausible biomarker for sperm quality and function.

Several investigations have correlated sperm mtDNA copy number with different semen parameters in both fertile and infertile men (May-Panloup *et al*, 2003; Song and Lewis, 2008; Zhang *et al*, 2016; Wu *et al*, 2019a; Wu *et al*, 2019b). However, semen parameters are generally poorly predictive of reproductive outcome, which, in the case of infertile couples, is the birth of an infant. Only one prior study has evaluated the relationship between sperm mtDNA CN and assisted reproductive technology (ART) outcomes, but only with respect to laboratory parameters, including fertilization rate and embryo morphology (Wu *et al*, 2019b). No previously published investigation has reported live birth outcomes associated with sperm mtDNA CN values in infertile couples.

The aim of this work was to determine whether sperm mtDNA copy number is predictive of live births after embryo transfer as well as laboratory parameters (specifically fertilization, embryo development to the blastocyst stage, and euploidy rates) in an infertile population undergoing ART with IVF/ICSI. We hypothesized that if sperm mtDNA copy number is associated with sperm quality, then mtDNA CN should also be correlated with fertilization, usable blastocyst development, euploidy and live birth rates.

Materials and Methods

Study Population

Consent was prospectively obtained for use of de-identified, otherwise discarded whole sperm material between 2007 and 2013. Unique sperm samples were selected that were used to create transferred blastocysts. Only couples undergoing their first IVF cycle and embryo transfer at our center were included. Couples with severe male factor infertility were excluded. Additionally, samples from IVF cycles utilizing surgical or cryopreserved sperm, conventional insemination, or day 3 embryo biopsy were

excluded. Cycles in which the female patient had a BMI > 35 or failure of endometrial proliferation \geq 6mm were also excluded. The primary outcome was live birth rate. Secondary outcomes included fertilization rate, development rate of usable blastocysts, and blastocyst euploidy rate. A sample size of 2,000 patients was chosen to achieve > 80% power to detect a 10% difference in live birth rates, which was felt to be clinically significant. Differences were considered to be significant if $p < 0.05$.

Routine Ovarian Hyperstimulation and Laboratory Procedures

Patients underwent routine controlled ovarian hyperstimulation with ultrasound and serum steroid monitoring every one to three days. When at least two ovarian follicles reached 17mm in diameter, final maturation of the oocytes was achieved using either chorionic gonadotropin for injection and/or gonadotropin-releasing hormone agonist. Approximately 36 hours later, vaginal oocyte retrieval was performed. Oocyte denudation was performed after retrieval, as ICSI was utilized in all included cases. ICSI was performed approximately five to six hours after oocyte retrieval. The inseminated oocytes were placed into incubators overnight. The next morning, all inseminated oocytes were assessed for the formation of two pronuclei, signifying fertilization. Zygotes were placed back into culture until day 3 post-retrieval, at which time the embryos were evaluated and laser-assisted hatching was performed. Embryos were cultured until day 5, at which time they were assessed for development into a blastocyst. If a blastocyst appeared to be expanded with an adequately cellular inner cell mass (ICM), then it was considered usable and soon vitrified for later use. For patients electing PGT-A, blastocysts considered to be usable underwent trophectoderm biopsy with subsequent vitrification.

Evaluation of Relative Mitochondrial DNA Copy number

Sperm DNA was isolated with the use of the Qiagen DNeasy mini kit. Mitochondrial DNA copy number was evaluated using a quantitative real-time polymerase chain reaction (qPCR) employing two mtDNA TaqMan® Copy Number assays (16SrRNA and MajArc) and normalized to a multi-copy Alu nuclear DNA target for relative quantitation (Fragouli *et al*, 2017). The difference in number of cycles reached by the sequence of interest and the reference sequence is referred to as the delta cycle threshold (Ct). The delta Ct, a measure of the relative mtDNA content, was calculated as the difference between the

assay Ct value (16SrRNA or MajArc) and the internal nuclear control Ct value (AluYB8). The delta Ct values were centered by subtracting the per-plate median delta Ct value. The two mtDNA assays were concordant, so results were averaged. Results were expressed in relative mtDNA quantity for ease of interpretation, which was the negation of the adjusted delta Ct values. Therefore, sperm relative mtDNA quantity remained in log scale. For the purposes of validation, the assay was performed on a human fibroblast cell line (Coriell Cell Repository ID GM01359) treated with ethidium bromide (25 ng/mL in full growth medium) in order to create samples expected to possess fewer mitochondria, and therefore, lower levels of mtDNA.

Preimplantation Genetic Testing for Aneuploidy Screening

For patients requesting preimplantation genetic testing for aneuploidy (PGT-A), trophectoderm biopsies were examined for the presence of aneuploidy using a quantitative real-time polymerase chain reaction-based assay (qPCR) as previously described by Treff *et al*, 2012.

Statistical Analysis

Patient and cycle characteristics for the study cohort were determined. Linear regression was used to assess the relationship between relative sperm mtDNA value, total motile sperm count, paternal age and days of abstinence. The total motile sperm count was obtained by multiplying the semen volume by the concentration and percentage of rapidly progressive and slowly progressive motile spermatozoa. Mixed effects logistic regression models were used to explore relationships between sperm mtDNA CN and fertilization, usable blastocyst development, euploidy and live birth rates. Fertilization rate was calculated by dividing the total number of bi-pronuclear zygotes by the total number of metaphase II oocytes inseminated per cycle. Usable blastocyst development rate was calculated by dividing the total number of blastocysts deemed usable (i.e., expanded blastocyst with adequate trophectoderm and inner cell mass cellularity), which were either cryopreserved or transferred, by the total number of bi-pronuclear zygotes per cycle. Euploidy rate was calculated by dividing the total number of euploid embryos by the total number of embryos deemed usable and, therefore, biopsied per cycle. Covariates for

each regression model included female age and sperm pre-wash motility. All statistical analyses were performed using the statistical computing program, R (version 3.4.3).

Ethical Approval

Advanced approval for the study was obtained from the institutional review board, with individual written informed consent from each patient for the use of otherwise discarded, de-identified, whole sperm material for research purposes, as well as for the review of the medical chart (RMA-00-02). All patients during the study time period were presented with the option of donating otherwise discarded biologic sperm material exclusively for research use.

Results

Study Participants

A total of 2,062 unique sperm samples used to create transferred embryos were identified from patients granting consent. The mean maternal age at retrieval was 34.3 years ($SD \pm 4.8$), mean paternal age was 37.6 years ($SD \pm 5.8$), and average pre-wash sperm concentration of 49.8 million/mL (± 40.8). Of all transfers, 71% were frozen/warmed embryo transfers and the remainder were fresh embryo transfers. The mean number of embryos transferred was 1.2 ($SD \pm 0.8$), and overall the live birth rate was 64% (Table I). The proportion of women age < 35 years at the time of oocyte retrieval was 44%; 28% of women in the study population were 36-37 years at the time of oocyte retrieval, 21% were 38-40 years of age and 7% were 41-42 years of age at the time of retrieval. Preimplantation genetic testing for aneuploidy (PGT-A) via trophectoderm biopsy with subsequent euploid embryo transfer was performed in 428 (20.8%) of all study cycles. PGT-A cycles occurred between 2011 and 2013. All other included IVF cycles utilized ICSI but did not utilize PGT-A.

Outcomes

The mean relative mtDNA value for each sperm sample was determined. Lower relative sperm mtDNA quantity was associated with increased pre-wash sperm motility ($p > 0.001$). No association was

identified between sperm mtDNA CN and paternal age ($p=0.40$) or days of abstinence ($p=0.94$) prior to sample collection while accounting for maternal age and pre-wash motility (Figures 1a and b).

Additionally, no association was seen between sperm mtDNA CN and fertilization ($p=0.40$) or usable blastocyst development rates ($p=0.36$) when accounting for maternal age and pre-wash motility (Figures 2a and b). With respect to cycles utilizing PGT-A, there was no significant association between sperm relative mtDNA quantity and whole chromosome euploidy rate per embryo ($p=0.10$). Further investigation of whole chromosome euploidy rates of the first and fourth quartiles with sperm mtDNA CN was subsequently performed, but also failed to reveal a statistically significant association (Figures 3a and b). Finally, no difference in live birth rates was identified by sperm relative mtDNA quantity when adjusting for maternal age and pre-wash motility ($p=0.42$) (Figure 4).

Discussion

Our analyses reveal that lower sperm relative mtDNA copy number is associated with increased sperm motility, which corroborates the findings of several other investigations, who have previously evaluated the relationship between sperm mtDNA CN and semen parameters. Although other authors have extrapolated such findings to prognosticate reproductive outcomes, no other study has evaluated live birth as a primary outcome. In this first analysis of the relationship between both clinical (i.e., live birth rates) and laboratory outcomes (i.e., fertilization, usable blastocyst development, and blastocyst euploid rates) with sperm mtDNA CN from infertile patients undergoing IVF with ICSI, no correlation was identified. Therefore, sperm mtDNA CN testing was not found to add any additional prognostic value toward laboratory or clinical outcomes when utilizing IVF with ICSI.

A relationship between elevated sperm mtDNA CN and abnormal semen parameters has been described in both fertile and infertile men (Song and Lewis, 2008; Zhang *et al.*, 2016). Several theories have been proposed to explain the physiologic basis for an increase in mtDNA CN in abnormal sperm. For example, it has been postulated that high levels of reactive oxygen species may slow the removal of mitochondria and their genome during sperm maturation. Alternatively, it has been proposed that increased production of mtDNA may occur in the presence of defective mitochondria as a compensatory response. Finally, some experts suggest that abnormal spermatogenesis may lead to dysregulated

apoptosis, resulting in unusually elevated mtDNA content (Moyes *et al.*, 1998; Lee *et al.*, 2000; May-Panloup *et al.*, 2003).

Although such theories provide plausible justifications for a relationship between elevated mtDNA CN and impaired sperm quality, our findings failed to show that such an effect translated to suboptimal embryologic parameters, as evidenced by lack of difference in fertilization rates, usable blastocyst development rates, and euploidy rates. Additionally, an absence of impact of sperm mtDNA CN on clinical outcomes was also observed, as evidenced by no difference in live birth rates when sperm samples with lower and higher mtDNA values were used to create the transferred blastocysts. Our results may be explained by either a genuine lack of influence on the developing embryo by sperm mtDNA CN or possibly related to ICSI, via selection of a single sperm for insemination. In the case of the former, elevated mtDNA CN would be present in the inseminated sperm but would fail to have an effect on embryo development, likely due to the previously described rapid degradation of sperm mtDNA occurring post-fertilization (Cummins *et al.*, 1998; Hajjar *et al.*, 2014). Alternatively, the single sperm cell selected for insemination at the time of ICSI may have had lower than the average mtDNA CN compared to the sample from which it was derived. This explanation is equally reasonable, as sperm selected for ICSI are motile when available, and motility is inversely associated with sperm mtDNA CN. It is therefore possible that selection of a motile spermatozoa for ICSI reduces the odds of selecting sperm with unusually elevated mtDNA CN. However, conventional (IVF) and in vivo insemination also require motile sperm. Thus, further evaluations are necessary to delineate whether sperm selection at the time of ICSI similarly alters the likelihood of choosing sperm of reduced mtDNA content as compared with conventional insemination.

The major strength of this study is its large sample size, allowing for power to detect a difference in a relevant outcome of interest, e.g., live birth. Limitations of this study include the assumption that quantification of the mean mtDNA content of a population of sperm is representative of the individual spermatozoa selected for insemination. Furthermore, it is unknown whether the overall sperm mtDNA CN is consistent between different samples collected from the same patient and, therefore, a reproducible test. Finally, the time frame of this investigation (e.g., 2011 to 2013) may also be a limitation. Although study cycle characteristics were found to be similar to current cycles in our system, occult differences between

past and present laboratory and clinical environments may exist, limiting the generalizability of our conclusions in ways that are not readily apparent.

Although sperm relative mtDNA CN was not found to be prognostic of IVF/ICSI cycles in this infertile population, it is plausible that sperm mtDNA CN, or the lack thereof, has a role in embryo development and reproductive competence. However, many questions in this area remain unanswered. Future directions of research may include developing a deeper understanding of the underlying etiology of elevated mtDNA value in low motility sperm. Additionally, investigating the effects on offspring derived from embryos created with such sperm may be warranted. Finally, identifying whether a relationship exists between mitochondrial number and mtDNA CN within gametes and/or embryos would be useful in furthering our understanding in this area.

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Author's Roles

AW Tiegs: Study execution, manuscript drafting, revising, interpretation of data

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JM Fransiak: Manuscript revision

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Conflict of interest

The authors declare that they have no conflict of interest.

Compliance with ethical standards

Advanced approval for the study was obtained from the institutional review board, with individual written informed consent from each patient for the use of otherwise discarded, de-identified, whole sperm material for research purposes, as well as for the review of the medical chart.

Informed consent

Informed consent was obtained from all participants included in the study.

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