Title page

Sperm mitochondrial DNA copy numbers in normal and abnormal semen analysis: a systematic review and meta-analysis.

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Abstract

Background:

Normal mature sperm have a considerably reduced number of mitochondria which provide the energy required for progressive sperm motility. Literature suggests that disorders of sperm motility may be linked to abnormal sperm mitochondrial number and function.

Objectives:

To summarize the evidence from literature regarding the association of mitochondrial DNA copy numbers and semen quality with a particular emphasis on the spermatozoa motility.

Search strategy:

Standard methodology recommended by Cochrane.

Selection criteria:

All published primary research reporting on differences in mitochondrial DNA copy numbers between the sperm of males with a normal and abnormal semen analysis.

Data collection and analysis:

Using standard methodology recommended by Cochrane we pooled results using a random effects model and the findings were reported as a standardised mean difference.

Main results:

We included 10 trials. The primary outcome was sperm mitochondrial DNA copy numbers. A meta-analysis including five studies showed significantly higher mitochondrial DNA copy numbers in abnormal semen analysis as compared to normal semen analysis(SMD 1.08, 95% CI 0.74-1.43). Three other studies not included in the meta-analysis showed a significant negative correlation between mitochondrial DNA copy numbers and semen parameters. The quality of evidence was assessed as good to very good in 60% of studies.

Conclusions:

Our review demonstrates significantly higher mitochondrial DNA in human sperm cells of men with abnormal semen analysis in comparison to men with normal semen analysis.

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Keywords

Mitochondrial DNA, sperm motility, abnormal semen parameters

Capsule:

There is significantly higher mitochondrial DNA in human sperm cells of men with abnormal semen analysis in comparison to men with normal semen analysis

Introduction

Mitochondria are one of the fundamental cell organelles providing the cell with energy in the form of adenosine triphosphate (ATP) by the process of oxidative phosphorylation (OXPHOS). The amount of mitochondria varies with cell type and function (1). The process of spermatogenesis results in a drastic decrease in the number of mitochondria (2). This pre-fertilisation reduction in sperm mitochondrial content is aimed to reduce/eliminate paternal mitochondrial transmission in conjunction with other post-fertilisation mechanisms resulting in uniparental inheritance. Mature sperm are thought to contain between 22–75 mitochondria providing the energy required for progressive sperm motility (3). Sperm motility is dependent on the energy provided by OXPHOS (4).

It has been suggested that male infertility and disorders of sperm motility may be linked to abnormal sperm mitochondrial number and function. Male infertility has been reported in men with mitochondrial disorders (5). Also, associations between abnormalities of sperm mitochondrial DNA and abnormal sperm parameters have been reported (6). Early reports available on mitochondrial DNA quantification in mammalian sperm present widely varying results (2, 7). In humans, few studies report the association of mitochondrial DNA copy number (mtDNAcn) with sperm motility and other semen characteristics (8-10).

The aim of this review is to summarize the evidence from literature regarding the association of mtDNAcn and semen quality with a particular emphasis on the spermatozoa motility. This aims to guide clinical practice and give direction for future research.

Materials and methods

Eligibility criteria:

Our search aimed to identify all published literature reporting on differences in mtDNAcn between the sperm of males with a normal semen analysis and males with abnormal semen analysis. All types of studies published as primary research were included for the review. We included only those studies published in the English language, published as full manuscripts (not abstracts) and those involving humans-only. We included studies where semen samples were analysed based on either the WHO 1999 or 2010 criteria. The methodology for undertaking the review was developed following recommendations of CRD's guidance for undertaking reviews in health care (Centre for Reviews and Dissemination) (11). Results were reported in accordance with PRISMA guidelines (12). The review was prospectively registered with PROSPERO (CRD42019118841).

Assessment of study quality and the risk of bias:

Assessment of study quality was done using the Newcastle-Ottawa Scale (NOS) modified for cross sectional studies. Further modification was used as only non-interventional observational studies were included. We conducted a comprehensive search for eligible studies in order to minimize the impact of reporting bias.

Main outcome measures

The primary outcome measure was sperm mitochondrial DNA copy numbers.

Data sources

DP and FD independently screened and identified studies which were relevant for the review. Standard Cochrane methodology was followed comprising electronic searches and hand searching. Embase Classic and Ovid MEDLINE were searched on December 07, 2020. The study period was from 1946 to 2020. We used the controlled vocabulary of Medical Subject Headings (MeSH) terms "Male Infertility" and 17 additional keywords related to or describing the participants and/or outcome (e.g. asthenospermia, oligospermia, sperm quality). The detailed search strategy for MEDLINE and Embase can be found in Supplementary Materials Appendix S1. We updated our search by re-conducting the search 1 month prior to submission of the review for publication. The reference lists of relevant articles were screened to identify additional studies.

Data collection

DP and FD independently screened the title, abstract and keywords (ti,ab,kw) of the retrieved articles. The full text of potentially suitable articles was retrieved. From these

suitable articles were finalised for inclusion for the review. Agreement regarding potential relevance was reached by consensus. Inconsistencies were discussed among the reviewers and resolved by discussion with a third author. Conference abstracts were excluded from the quantitative analyses to avoid publication bias.

DP and FD reviewed all selected articles and extracted relevant data regarding study characteristics independently. Data were collected on a bespoke data collection Excel sheet where data were collected for study design, methodology, participant characteristics and outcome variables. Multiple publications of a single study were pooled together under a single study ID. All identified references were exported to EndNote X 8.2 for Windows, where the list of publications was scanned for duplicates.

Data analysis and synthesis:

The pooled estimates for the outcome were presented as Standardised Mean Difference (SMD) with 95% confidence intervals using the random effects model and inverse variance method. Statistical significance was assumed when $p<0.05$. In case where the information in the studies was not reported in the way appropriate for our data extraction, the authors were contacted. We were able to get this information for the study Tian 2014 (10), and have updated our analysis accordingly. Studies were excluded from meta-analyses if the data were presented using correlation analyses and without dividing the semen of patients into categories (normal/abnormal) or using different laboratory methodology.

Results

General characteristics of studies

Results of the search:

The search of the two electronic databases retrieved 373 full text articles after removal of duplicates. No further articles were retrieved by hand searching of the reference lists. After screening of the titles and abstracts, the full text of 19 studies were retrieved for further review. 10 of these studies were selected for the systematic review and 9 excluded. Of the 10 selected studies, five were suitable for meta-analysis and included for quantitative synthesis. The search and selection process are documented with a PRISMA flow chart in Figure 1 and the list of included and excluded studies with reasons for exclusion provided in Supplementary Materials, Table S1.

Included studies:

The characteristics of the included studies are detailed in Supplementary Materials, Table S2.

Study design and setting:

The 10 studies included in this systematic review were all single-centre observational cross-sectional studies conducted across eight countries. Only five studies had a sample size of greater than 100 participants which we feel is satisfactory for providing good quality evidence. The largest study was conducted by Diez-Sanchez 2003 (13) from Spain and included 440 participants.

Participants:

Eight of the 10 studies recruited participants from fertility clinics, denoting a convenience sampling strategy, with only one of these studies recruiting healthy volunteers as controls. Two studies recruited volunteer donors for their studies. Only five of the 10 studies accounted for confounding factors such as age, BMI and lifestyle factors in the design and/or analysis stage of their studies. Hence, the comparability of the participants in the included studies or within study groups cannot be estimated. The study group for five of the 10 studies included in the meta-analyses comprised of men with abnormal semen analysis. The criteria for abnormal semen analysis however showed significant heterogeneity. Some studies reported results based on the WHO 1999 criteria whereas others used the WHO 2010 criteria. Some studies included men with only reduced sperm motility and normal sperm counts as the abnormal semen analysis for the study group. Few studies divided the abnormal results into subgroups, these however were dissimilar amongst the studies and hence it was not possible to conduct a subgroup analysis for a pooled estimate.

Outcome:

All studies reported the mtDNA/nuclear DNA ratio expressing the average mitochondrial DNA copy number per sperm. The values for the ratio variables differed considerably between the studies, which might be explained by the methodological differences in interventions. The concept however remained constant across the studies. The ratios were compared between patients with normal and abnormal WHO semen criteria. Two studies compared mtDNA content between sperm cells from the same semen sample in addition to mtDNA content from the different patients (May-Panloup 2003; Diez-Sanchez 2003).

Five of the 10 included studies reported the primary outcome as a mean +/- SD/SEM. Two studies reported the median + IQR/range. One study which reported the mean without a SD had to be excluded from the meta-analysis (9). Three studies reported the correlation between sperm mitochondrial DNA with sperm parameters rather than differences amongst defined groups with normal and abnormal semen parameters(13-15). One study used a

different methodology for estimation of DNA (16). These studies were not included in the meta-analysis.

Assessment of outcome:

The method of mtDNAcn assessment is a multistep process and varied amongst studies. The time range between the first study and the last was 16 years, which can impact on the technical differences between the former and the latter experiments. In general, to quantify mitochondrial DNA copy number, polymerase chain reaction (PCR) assay using specific primers to mitochondrial genes was used in the studies. To quantify the number of spermatozoa in the sample, nuclear DNA was determined. The relative mtDNA copy number was identified based on the mtDNA/nuclear DNA ratio.

The first step toward mtDNA quantification is a semen sample purification from the other cell types, i.e., leukocytes, round cells, epithelial cells, and miscellaneous debris. The fresh semen samples were purified using various methods such as a combined density gradient centrifugation and a swim-up method (May-Panloup 2003) (8), only-Percoll density gradient centrifugation (Amaral 2007; Bonanno 2016; Wu 2019) (14, 17, 18), Ficol-Paque fractionation (Kao 2004) (16), or without washing at all (Faja 2019) (19). Tian (2014) (10) used cryopreserved semen samples that have been thawed with subsequent washing in phosphate-buffered saline (PBS) and sperm-wash buffer. The absence of round cells in sperm preparations was checked by light microscopy in all studies. In two studies, semen samples underwent osmotic shock to eliminate the non-gamete cell component (Kao 2004, Faja 2019).

Various commercial DNA isolation kits were used by eight of ten included studies according to the manufacturer's instructions to extract total DNA. In two studies (Kao 2004 and Diez-Sanchez 2003) the total DNA was extracted using the phenol-chloroform method. May-Panloup 2003, Diez-Sanchez 2003, Kao 2004, Amaral 2007, and Song 2008 reported supplementation with dithiothreitol (DTT) and proteinase K to dissociate mitochondria from the mitochondrial sheath and disrupt the sperm nucleus disulfide bonds (20). The other three studies used only proteinase K as part of commercial DNA isolation kit (Tian 2014, Wu 2018, Faja 2019) or there was not any specification in the study or manufacturer's manual (Bonanno 2016, Zhang 2016).

Amplification of nuclear and mitochondrial genes was carried out by real-time PCR (qPCR) in eight of ten studies to determine the amount of mtDNA relative to nDNA. The mtDNA copy number per sperm cell was measured relative to a nuclear gene, for example, ß-globin gene (May-Panloup 2003, Kao 2004, Amaral 2007, Tian 2014), Glyceraldehyde 3-phosphate dehydrogenase - GAPDH gene (Song 2008, Bonanno 2016, Zhang 2016), calicin gene (Faja

2019), or gene of RNase P (Wu 2019). In the study of Diez-Sanchez (2003) mtDNAcn was determined by slot-blot hybridization using specific mitochondrial (16S rRNA) and nuclear probes (to 18S human rRNA). Kao and colleagues (2004) used a hot-start concurrent PCR to determine the amount of mtDNA relative to nuclear DNA. PCR products of mitochondrial ND1 and nuclear genes ß-actin were blotted onto a membrane for relative intensity measurement. This ratio was an index of the relative amount (copy number) of mtDNA with respect to nuclear DNA.

Melting curve analyses were done to verify the accuracy and specificity of genes amplification. Serial dilutions of recombinant plasmids containing mtDNA insert were used as the external standard to establish a quantitative reference for mtDNA quantification (May-Panloup 2003, Diez-Sanchez 2003, Kao 2004, Song 2008, Tian 2014;,Bonanno 2016). In the study of Amaral 2007, the external standard for qPCR was double-stranded DNA molecules. The linearity of the standard curve indicated the efficiency of PCR over the whole process.

The relative mtDNA copy number was calculated using the formula mtDNAcn/nuclear gene copy number in all studies. In the study Faja 2019, fluorescence data were converted to cycle threshold (Ct) for each gene. The relative mtDNA content was obtained by calculating the ΔCt (ΔCt=CtCOII − Ctcalicin) for each sample and applying the exponential function $2^{-\Delta Ct}$ (17).

Quality of evidence and the risk of bias:

The quality of evidence assessed by the NOS was good to very good in 6 of the 10 studies, and no study was considered unsatisfactory. 70% of studies were downgraded due to the use of convenience sampling and 50% for small sample sizes included. The results are summarized in Supplementary Materials, Appendix S2.

Synthesis of the results:

Of the 10 studies reporting on differences in sperm mitochondrial DNA, five studies with 530 participants were included in the quantitative meta-analysis (Amaral 2007, Bonanno 2016, Faja 2019, May Panloup 2003, Tian 2014)(8, 10, 17-19). The results are seen in Figure 2. A significant difference in sperm mitochondrial DNA copy numbers was seen between the normal and abnormal semen analysis groups (SMD 1.08, 95% CI 0.74-1.43). All five included studies reported higher sperm mitochondrial DNA copy numbers in abnormal semen samples as compared to normal semen samples. Significant statistical heterogeneity was noted (Tau^2=0.09, Chi^2=10.23, df=4, p< 0.04, I^2=61%). Three studies reported a significant negative correlation between mitochondrial DNA copy numbers and semen parameters (8, 14, 15).

Subgroup analysis

No subgroup analysis was done due to dissimilar subgroups of abnormal semen analysis.

Discussion

Main findings:

Our systematic review and meta-analysis of data showed a significant difference in sperm mitochondrial DNA copy numbers in human sperm cells with abnormal parameters in comparison to normal sperm cells. Three studies reported a negative correlation between mtDNAcn and (1) sperm motility (Tian 2014, Bonano 2016, Faja 2019), (2) total sperm count (Song 2008), (3) sperm concentration per mL (Amaral 2007, Tian 2014) and (4) morphology (Amaral 2007) between patients with abnormal semen parameters and control groups. Animal studies also support the findings of this review, that increased mtDNAcn is associated with decreased total sperm motility (21) A single study reported a large effect size but an opposite direction of effect (16). This could be attributed to a different method for estimation of DNA.

Semen is a complex fluid containing different cell types. Besides leucocytes, immature germ cells, white blood cells, and epithelial cells, there is variation in sperm population regarding motility and morphology. Namely, motility within one semen sample can be graded as progressive, non-progressive, and immotile. In order to eliminate seminal plasma, diploid cells of different etiology, and separate sperm according to motility and morphology semen purification was applied in nine of ten studies as described in the outcome assessment section. Hence, the assessment of mtDNAcn was done using sperm cells selected from the best fraction of semen population between different men. Moreover, in two studies by Diez-Sanchez (2003) and May-Panloup (2003), they compared mtDNA content between sperm cells from different populations of the same sample without taking into account the initial sperm quality. It was found that cells from the semen fraction of worse quality had higher mtDNA quantity than sperm cells from the fraction of better quality (8, 13).

The WHO criteria classifies abnormal semen analysis into three major groups; asthenospermia (A), oligospermia (O), and teratospermia (T) and their different combinations such as AOT, AO, OT, and AT (22). Our review indicates that those who have more than two abnormal criteria have in average increased number of mtDNA copies. Amaral 2007 analyzed the mtDNAcn between three male fertility groups: normal, with 1 or 2 sperm defects or more than to defects (AOT). The group including three defects (AOT) as low sperm number, decreased motility, and abnormal morphology statistically differed from the normal group ($P<0.01$) and from 1- or 2- defects group ($P<0.05$). Comparing all groups

one by one, there was a significant negative correlation between mtDNAcn/sperm concentration (R=-0.561, P<0.001) and mtDNAcn/sperm morphology (R=-0.467, P<0.002). At the same time, mtDNA content per sperm from the group with the only motility defect did not differ significantly from the sperm of normal group, but there was a trend towards correlation (R=-0.285, P=0.067). This is similar to the negative correlation of mtDNAcn and motility in the study of Tian 2014 (r=-0.37; P<0.001). This data also corresponds to the results of May-Panloup 2003 where semen with the only abnormal criteria (A or T or O) was not significantly different from the mtDNAcn in the normal group. However, highly significant difference was detected between patients with normal sperm and the group including multiple abnormalities (O, A, T, OA, AT, OAT) (P<0.0001) (8). The results of Song 2008 are are in agreement with the studies of Amaral 2007 and May-Panloup 2003 that mtDNAcn increased in the group of multiple abnormalities (AOT) compared with normal semen and patients with the only abnormal semen criteria (P<.05, Tukey test).

Two studies report mitochondrial DNA quantity specifically for asthenozoospermic patients in comparison to healthy men. In the study of Bonanno 2016, the analysis was performed in 37 patients with idiopathic asthenospermia, i.e., with a high percentage of sperm with low motility. The increased quantity of mtDNAcn was detected in 45.8% of patients, that correlated with high reactive oxygen species (ROS) production. At the same time, Faja 2019 reported mtDNAcn analyses on 63 asthenozoospermic samples with progressive motility less than 32%. There was a significant correlation between mtDNAcn and total motile spermatozoa (r=-0.51, P<0.001), sperm concentration per mL (r=-0.50, P<0.001), and total sperm count per ejaculate (r=-0.44, P<0.001). It is important to note that in the study of Faja 2019) there was no sperm purification with cell selection regarding motility or morphology. That implies that analysis was done on the general sperm population that might result in a higher level of correlation rate in comparison to studies with sperm selection through semen purification.

Strengths and limitations

To our knowledge, the review is the first to assess the human sperm mitochondrial DNA copy numbers. Despite the general trend between the studies, there is a wide range of mtDNA quantities. Several possible aspects result in a wide variation of outcomes such as duplication of the mitochondrial genome in nuclear DNA, the use of inappropriate primes, the bias of dilution, the low efficiency of total DNA extraction (23). Among other things, accurate quantification of mtDNA depends on the residual contamination of somatic cells in the analyzed sample. Thus, Diez-Sanchez 2003 revealed a positive correlation between the percentage of round cells in the semen sample and the relative amount of sperm mtDNA (13). Considering the susceptible nature of mitochondrial DNA to degradation, there may be

deletions in the analyzed gene region due to oxidative stress. This may result from the presence of leucocytes which active producers of extracellular ROS in semen (24). Hence, it might be reasonable to determine mtDNAcn in sperm cells using several mitochondrial genes.

The study population may also affect the outcomes. It has been shown that the semen quality depends on the geographical region, as shown for the US and Europe (25, 26). Moreover, seasonal variation of sperm concentration and total sperm count has also been reported (26). All these factors may cause the mtDNA count variation in sperm cells.

Interpretation

The mechanisms behind the association of mtDNAcn and abnormal semen parameters are still unknown. Several explanations have been proposed. The mature human spermatozoa contains residual quantities of mtDNA which is decreased during spermatogenesis. Rantanen and Larsson proposed the hypothesis of mtDNAcn decrease during spermatogenesis through downregulation of Tfam proteins in spermatids, which is known to be the transcription and replication regulator of mitochondrial DNA (27-29). Adverse external factors or genetic issues may affect to the process of spermatogenesis to prevent this normal reduction in mtDNA. Sometimes, these changes might have a compensatory value; for instance, Jiang and colleagues demonstrated on the mouse model that the increase of mtDNAcn can improve a severe disease phenotype caused by mtDNA mutations in testis (30)(43). Hence, the level of normal mtDNA without mutation will be higher, but the mtDNA mutation load remains the same.

Based on the results mentioned above, the mtDNA copy number may potentially have a prognostic value for fertility and ART outcomes. A few studies presented the connection between mtDNAcn in sperm and clinical outcomes during ART procedures (13, 31-33) . For example, Tieg 2020 reveals no relationship between live birth rates, fertilization, usable blastocyst development, and blastocyst euploid rates with sperm mtDNAcn from infertile patients undergoing IVF with ICSI (28). It is possible that the sperm cell selected for ICSI had lower mtDNAcn than other cells from the same semen because of a heterogenic population of sperm cells. Simultaneously, Tieg's 2020 analysis has confirmed the association of lower relative mtDNAcn with increased sperm motility. Another study by Rosati 2020 revealed the association of mtDNAcn with lower pregnancy probability within 12 months and a longer time to pregnancy. The pregnancy probabilities decreased linearly with higher mtDNAcn (31). The association of mtDNAcn of sperm cells and early ART outcomes was also analyzed by Wu 2019. The results suggest that sperm with higher mtDNAcn may result in lower odds of embryo development to Day 3 and Day 5 (33).

Regardless of the effect on ART's clinical outcomes, the levels of mtDNAcn may be used as a predictor of spermatogenic dysfunction in men. Gabriel and colleagues suggested mtDNAcn as an indicator of spermatogenesis's efficiency based on the significant decrease of mtDNA quantity after varicocelectomy (34). Furthermore, the mtDNA content may play a role of a bioindicator of environmental pollutants such as an air pollutants exposure (35), polycystic aromatic hydrocarbons (PAHs) resulted in reproductive health problems (36), and synthetic organic chemicals as monocarboxy-isononyl phthalate, which were positively associated with mtDNAcn (37). Prolonged exposure to SO_2 is negatively associated with mitochondrial quantity (35). Another study by Luo (2012) revealed the increase of mtDNAcn with hypoxic conditions at high altitudes (5.300m) (38). Given the reversible effect on sperm quality and mtDNA content of environmental and some external factors such as sexual abstinence before the collection of the semen, heating, cigarette smoking, and lifestyle, Wu 2019 suggests that mtDNAcn might be suited as an indicator of male reproductive status on the ground of consecutive diagnoses rather than a single abnormal sample (14).

Conclusion

In this review, we have demonstrated a significantly higher number of mtDNA in human sperm cells of men with abnormal semen analysis in comparison to men with normal semen analysis. It is important to note that the quantity of mtDNA rises with the increase in semen abnormal parameters. Besides, the heterogeneous sperm cell population in the semen creates sperm variation of mtDNA copy number within the same sample. These findings would seem to suggest the predictive value of mitochondrial DNA quantification for male reproductive status assessment.

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Disclosure of interests

None declared. Completed disclosure of interests forms available to view online as supporting information.

Contribution to authorship

DP and PB contributed equally to the conception, planning, execution, analysis, writing and final approval of the manuscript. FD contributed to literature search, study selection, data

extraction and assessment of study quality. PBa revised the article critically for important intellectual content. GA contributed to the conception and planning, and revised the article critically for important intellectual content.

Ethical approval

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