Innovative Mutation of ATPase-8 Gene (8378 A > G) of Sperm Mitochondrial DNA of Infertile Males with Asthenozoospermia

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Abstract

Objectives: A spermatozoa-mitochondrial DNA (Sp-MtDNA) rearrangement is one of several etiologies of male infertility. The molecular base of this defect must be clarified to inspect the cause of diminished sperm fertility. This work has been designed to evaluate the Sp-MtDNA mutations as a candidate issue for males'-infertility. **Methods:** The study included 56 asthenozoospermic and 30-fertile males. A semen sample was collected from all participants. Direct sequencing was performed after the mitochondrial ATPase-8 gene (MtATPase-8) was amplified by PCR. **Results:** A novel nucleotide substitution was recognized significantly in the *MtATPase-8* of all patients in progressive and non-progressive motility sperm cells, but absent in the normozoospermic controls. One missense novel substitution 8378A>G in the *MtATPase-8* detected characterized by alteration of amino-acids "Asn to Asp". This substitution was persistent in the ≤ 5% group of sperms-motility, which was 7.5%, with lower frequencies in other sperms-motility groups. Non-progressive motility sperm portions were found to have the highest frequencies of the identified mutations, which had isolated from a 40% gradient in the ≤ 5% sperm-motility group. The latter represented 7.5% for 8378A>G mutation, instead of progressively motile sperms isolated from the 80% gradient portion.

Conclusion: In the current work, the mutation of Sp-MtDNA reveals a vital role in fertility and sperm motility attenuation, which are significant elements of male fertility. Moreover, in infertile men, the incidence of Sp-MtDNA novel mutation is highly frequent.

Keywords: infertility, asthenozoospermia, mitochondrial DNA, spermatozoa, sperm motility.

Introduction

Infertility is a male or female reproductive disorder expressed by a failure to attain conception in 12 months or more post-regular non-protected sexual intercourse (1). Infertility is a principal clinical and social concern that affects individuals universally (2, 3). It is presumed that around 60-80 million couples have complained of infertility globally, based on the World Health Organization reports (4). The male factor forms about 40% of infertility cases (5,6). Male infertility is associated with oligoasthenozoospermia or asthenozoospermia (A-zo), which may be observed in patients suffering from typical Sp-MtDNA abnormalities. However, the precise pathogenesis of A-zo is uncertain (7). These abnormalities could include point mutations

or multiple microdeletions of Sp-MtDNA (8). The sperm cells with microdeletions of Sp-MtDNA had correlated with weakened motility and fertility of the sperms. Nevertheless, research has revealed that Sp-MtDNA point mutations, single nucleotide polymorphisms (SNP), and haplogroups can critically affect qualitative semen properties (9). Asthenozoospermia is abnormal sperm motility and can induce infertility among males in 19% of cases (10). Isolated A-zo had been reported in 24% of infertile males (11), which might be due to sperm dysfunction, extended intervals of sexual abstinence, incomplete seminal tract obstruction, varicocele, infection, or heritable factors (12-14). Nevertheless, in some instances, A-zo might be idiopathic, and no specific cause is discernable by routine tests (15). The rate of MtDNA-mutation is several times more than nuclear DNA (16), which might be due to many pathogeneses including asexual replication, deficient repair apparatus, and absent protective histones, besides much closeness from the sites of free radical generation. It was found that there are nearly 70-80 mitochondria in the mid-piece of mammal sperms. It had established that there is a single copy of Sp-MtDNA in the mid-piece of every sperm mitochondria (9,17). The Sperm present in a state of oxygen inconsistency and necessitate high energy to provide their post-ejaculatory activity (18). Therefore, accidental attacks on the naked Sp-MtDNA by reactive oxygen species (ROS) or free radicals induce oxidative hypoxic damage or mutations to the MtDNA that can cause infertility (17).

To our best knowledge, no accessible data about the association between Sp-MtDNA variations and A-zo had previously been reported in Babylon. The present investigation aims to screen mutations in the Sp-MtDNA gene that affect sperm motility.

Material and methods

Selection of study participants:

A total of 56 males with A-zo attended the physicians in Teba Center for *in-vitro* fertilization and treatment of infertility in Babylon, Iraq. Thirty healthy men were selected as a control group. Sampling was completed throughout a timeframe from June 2019 through October 2019.

Inclusion criteria: Any male partners with a minimum one-year history of infertility, with the following criteria: a minimum of 2-semen analyses at an interval of 6 weeks, had to show A-zo, total sperm count is 40 $x10^6$/ml sperms or more, sperm motility in less than 50% of spermatozoa exhibiting (grades A and B) forward progression alternatively if sperm motility observed in less than 25% of spermatozoa with grade A motility (19).

Exclusion criteria: All the infertile males with a history of any of the following: testicular varicocele, accessory sex-glands infections, undescended testicle, prior operation of sex organs, sexually transmitted diseases, mumps infection, orchitis, testicular trauma, drug addicts, acute febrile infections, DM, thyroid diseases, vascular disorders, tuberculosis, and any long-lasting infection, had also been omitted from the study.

Seminal Fluid Evaluation

All the seminal analyses had been accomplished in the laboratories of the Teba Center. A semen sample was taken in a sterile container following a period of sexual abstinence of 3-5 days and 30 minutes of incubation at room temperature. Seminal investigation for all participants was conducted with "Computer Assisted Semen Analysis" (CASA) following the standards recommended by the WHO (19), which included assessments of sperm shape, motility, and quantity. The types of sperm motility were assessed and categorized as follows: "linear motility or rapid-progressive," "curvilinear motility or slow-progressive," "in-loco motility or non-progressive," and "absent motility."

Discontinuous Density Gradient Centrifugation using (Percoll method)

The Percoll method was utilized to separate the samples of all participants into two sets: slow motile sperm (non-progressive) and fast progressive

populations. This was achieved using a discontinuous density gradient of 40% and 80% PureSperm® (Nidocan, Flojellbergsgatan Mollndal, Sweden). Initially, two ml of the 80% PureSperm® gradient solution was aspirated with a micropipette fitted with a sterile tip and placed at the bottommost of a new sterilized pointed centrifuge tube. Subsequently, 2 ml of the 40% gradient solution was carefully layered on top. This process resulted in the formation of two distinct gradient layers. Next, 2 ml of freshly collected liquefied and examined semen was gently poured onto the 40% layer. The tube was then centrifuged for 20 minutes at 300 x g using an Eppendorf centrifuge (China).

Following centrifugation, several layers were shaped from top to bottom: the first layer consisted of seminal plasma; the second contained immotile or dead sperm, debris, WBCs, epithelial cells, and bacteria; the third layer included immature and senescent spermatozoa; the fourth layer contained non-progressive spermatozoa; and the final layer was a pellet comprising highly progressive motile sperm cells. Non-progressive sperm were retrieved from the interface between the 40% and 80% gradients, while fast-progressive sperm cells were gathered as a pellet at the bottom of the tube.

DNA Isolation

One milliliter of the sperm's suspension was transferred to a microtube of 1.5ml. DNA extract was obtained from the spermatozoa using the Diatome® DNA kits (Imogen, Russia), following the manufacturer's protocol, which involves lysis reagents containing guanidine thiocyanates. The concentration and purity of the extracted DNA were assessed using Nanodrops spectrophotometers (BIONEER, South Korea) at wavelengths of 260/280 nm

DNA Amplification

From the sperm of all participants, the *ATPase-8 gene* was amplified and sequenced. Primer sequences for the abovementioned genes were designed using the program Primer 3 Plus web (http://www.bioinformatics.nl/cgibin/primer 3 plus/primer 3 plus.cgi/). The polymerase chain reaction was performed in 0.2ml tubes by 5ng DNA, 0.5 µM of each primer, 200µM of dNTPs, 1x "PCR Phusion HF-Buffer" comprising 5 mM of MgCl2 , as well as 0.02 unit of "Phusion Hot-Start Π DNA Polymerase (New England -Biolabo)", (table-1).

DNA Electrophoresis

The amplified PCR products had run in electrophoresis on (1.50%) gel by drawing 4µl of PCR amplified products and mixed lightly with 1ul loading dye (Thermo scientific 6x DNA Loading Dye) as well as to positive control. A1ul of 100 base-pair DNA ladder ("Gene Ruler - Fermentas") was utilized as a molecular standard to check the amplified PCR product.

Sequencing Reactions and Electrophoresis

The PCR molecular products of *ATPase-8* were determined directly by sequencing in a 3700 ABI machine (Biosystem, USA). The master mix comprised "ONP185 reverse-primer". The thermal cycling protocol included: heat activation at 96^{o}C lasting one minute, then running 25 cycles at 96^{o}C/10s, 50^{o}C/5s, and 60-minute. Then, the cleaning of the sequence reaction was completed. Finally, an electrophoresis of PCR products was undertaken by the (Applied Biosystem) AB 3730 DNA Analyzer, USA.

Sequences Analyses of *MATPase-8* Gene

Sequencing data was inspected by software V-1.0 (Applied Biosystems, USA) and matched to the reference sequence at 'Genbank (accession No. NC 012920)" to indicate probable gene polymorphisms. The equivalent variations of amino acid with missense and silent polymorphism from MITOMAP. While those were not verified in MITOMAP ; the HmtDB. it:8080/hmdb/)" was utilized.

Statistical Investigations

The data statistical scrutiny was completed by SPSS (V-25/USA). The chi-squared test was used to compare the patient group with the healthy group and the P-value was considered significant if <0.05.

Results

The PCR products were sequenced after the mtATPase-8 gene had amplified (through conventional PCR), from Spermatozoal cells of the infertile males. The sequencing process was conducted using "Variant Reporter V-1.0" (Applied Biosystem, USA), and the gene variations were verified by aligning the Sp-MtDNA sequence with the revised Cambridge reference sequence. This alignment was performed by the MITOMASTER tool available online on the website at www.mitomap.org.

The sequences of the *MtATPase-8* gene were compared with a reference sequence, which has exposed a couple of novel nucleotide substitutions. These point mutations were found in the A-zo group, which included 56 infertile samples. The mutations were highly variable, with differing frequencies observed among the A-zo cases compared to the normozoospermic men.

The highest frequency of "novel heteroplasmic silent mutation" was noticed among a group of ≤ 5% sperm-motility (15%). The same percent was observed in *ATPase-8* gene 8899C>T and 8907C>T, individually, whereas the mutant genes were less frequent in the other sperm groups. Additionally, these events manifested at a minimal frequency within the normozoospermic individuals ($p < 0.05$). (Table 2). On the other hand, results had shown that the peak incidence of silent mutations in the nonprogressive motile sperms portions had isolated from a 40% gradient 8899 C>T, and 8907 C>T were 9 (13.5%) and 8 (12%), individually, instead of in progressively motile sperms, which were obtained from 80% gradient portion (Tables 3 & 4).

Table 1: Forward and reverse primers targeting sperm mitochondrial *MtATPase-8* gene used in the study

Direction	Sequence	Region	Site	Length
Forward Primer ONP25	5-CTACGGTCAATGCTCTGAAA-3	COXII	8161-8180	20nt
Reverse Primer ONP185	5-TACTATATGATAGGCATGTGA -3	COXIII	9239-9219	20nt

nt- nucleotide

Table-2: Conventional PCR thermal cycles for the amplification conditions

Cycling Steps	2-Steps Protocol		Cycles
	Temperature	Time	
Initial denaturation	94°C	4-minute	1
Denaturation	94°C	50 s	32
Annealing	55°C	50 s	
Extension	72°C	45 s	
Final extension	72°C	10-minute	1
	4°C	Hold	

s-second

Table-3: Frequency of Novel missense mutation 8738A>G in *MtATPase-8* gene of sperm-mitochondria astheno- vis normozoospermic participants

	Sperm Motility (%)						
WHO	Control (N=30)	0 – 5 (N=10)	6 – 10 (N=14)	11 - 15 (N=12)	16 - 20 (N=10)	21 - 25 (N=10)	Mean ± SEM (N=6)
Percoll	8378A>G mutation (Frequency / N)						
40% Fraction	0% (0)	7.5% (5)	4.5% (3)	4.5% (3)	3.0% (2)	1.5% (1)	2.33 ± 0.7
80% Fraction	0% (0)	1.5% (1)	1.5% (1)	1.5% (1)	0% (0)	0% (0)	0.5 ± 0.2
P value	0.036						
Total	0% (0)	9% (6)	6% (4)	6% (4)	3% (2)	1.5% (1)	
Gene	*MtATPase-8*						
Type (mutation)	Substitution (Missense)						
Amino acid change	Asn to Asp						
Mitomap reference	Novel						

SEM-standard error of mean, N-number

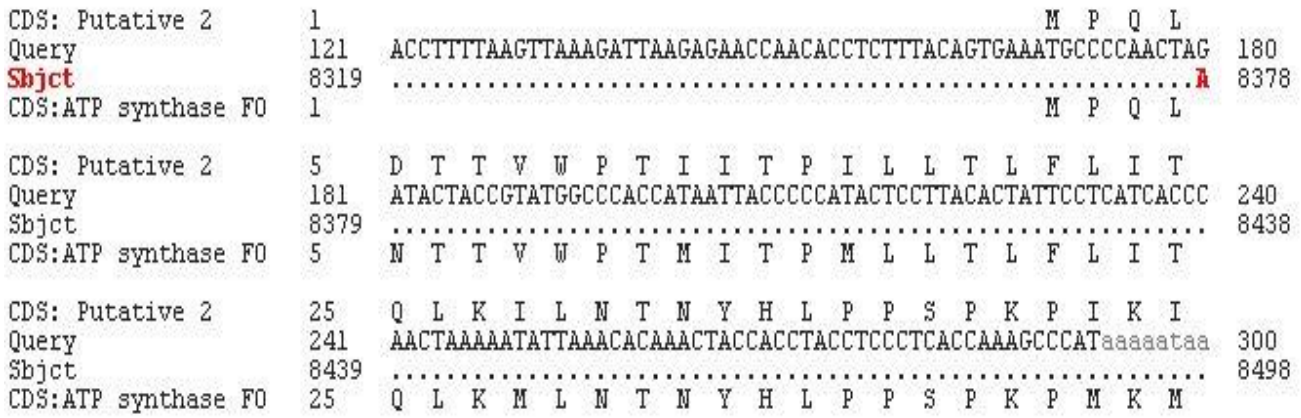

Figure-1: 8378 A>G mutation of sperm-mitochondria MtATPase-8 gene alignment with reference sequence by NCBI database

Discussion

Infertility and weakened fertility have been a worry through the ages and are also a significant clinical difficulty nowadays that affects 8–12% of couples universally. Almost 40–50% of infertile couples are caused by male factor (5,20). Nearly 2% of all males, will show suboptimal sperm parameters. It might be one or a combination of lower sperm count, and atypical sperm morphology/motility (21).

The results of the study indicate a high-frequent rate of the *MtATPase-8* gene mutations occurred among A-zo subjects rather than in control subjects. Our outcomes were consistent with a study that reported substantial nucleotide (nt) alteration in the (nt 8394) of the ATPase-8 gene in infertile males rather than the controls (22). The same study also observed a significant nt change in MtATPase-6 and 8 (ND 2, 3, 4, and 5) in the seminal fluid of males with infertility, which agreed with our results. Another study found significantly higher variations in the Sp-MtDNA genes: COXII, ATPase 6 and 8, ND 2, 3, 4, and 5 of spermatozoa from populations of Indian infertile males (23). The study reported a high frequency of point mutations (8557G>C) in the Sp-MtDNA ATPase-8 gene that arose in A-zo (24). In a study held in 2013, the researchers detected

innovative heteroplasmic-MtDNA missense-mutations (9387G>A) in the COXIII gene (8.8%) of three A-zo patients, which was not reported by the normozoospermic infertile or fertile males. This missense mutation replaces valine with methionine in preserved amino acids (25).

Normally, the cellular antioxidants able to remove the free cytoplasmic radicals and consequently avert oxidative cellular damage (26-29). Nonetheless, in sperm cells, and owing to cytoplasmic shedding throughout spermatogenesis, the lack of antioxidant enzymatic activity is frequently counterbalanced by the seminal elements of diverse sources (30). Two antioxidant enzymes, specifically catalase and glutathione peroxidase (Gpx), have been identified as significantly diminished in individuals experiencing infertility in contrast to their fertile counterparts (22). As Sp-MtDNA is the source of ATP synthesis via OXPHOS, also, it's also the place of reactive oxygen species (ROS) synthesis besides the initial site of ROS-induced injury (22). Seminal ROS can injure sperm by various pathways and is a common disorder observed in ≈ 2/3rd of infertile males. Several scholars have described high ROS values as an independent biomarker of male infertility (31-33). Data from other studies detected 3.3 fold rise in the ROS values in asthenozoospermic semen (34).

Furthermore, spermatozoa exhibiting Sp-MtDNA mutations may also demonstrate elevated levels of nuclear DNA fragmentation, which represents a premutagenic alteration that has the potential to elicit mutations in progeny (35). Consequently, the assessment of Sp-MtDNA is imperative for elucidating the underlying causes of motility disorders in oligoasthenozoospermic males. The instances that contain OS-induced Sp-MtDNA mutations possess a superior diagnostic capacity (36).

Three likely pathways of mutagenesis that cause Sp-MtDNA nt substitutions have been postulated. (1) The process of deamination affecting the nitrogenous bases of DNA. (2) The tautomeric shifts of protons occur within the structures of nitrous bases. (3) The hydrolysis of the glycosidic bond connecting DNA bases to carbohydrate moieties, occurring in the context of free radical-induced damage to the mitochondrial DNA polymerase gamma. Through the application of Quanto-chemical assessments, it has been demonstrated that the most substantiated mutagenic mechanism is the hydrolysis of the N-glycoside bond (37).

Oxygen species may similarly amend the DNA sequence of the mitochondrial gene (38). Thereby, reducing ATP synthesis and increasing the escape of ROS over the electron transport channel (39). Because throughout spermiogenesis, there will be more copies in Sp-MtDNA per cell (40) in the MtDNA-gene that may increase the sperm's free radicals, rather than other somatic body cells. Furthermore, increased Malondialdehyde in A-zo individuals may damage DNA through oxidation or covalent-binding (18).

Conclusion

In the current work, the mutation of Sp-MtDNA reveals a vital role in fertility and sperm motility attenuation, which are significant elements of male fertility. Moreover, in infertile men, the incidence of Sp-MtDNA novel mutation is highly frequent.

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Ethical Considerations

This study had been permitted by the authorities of the health institute at Babil Health Directorate of Babylon under the code (EC:1-4-2021, 4-721). As well, the American Psychological Association's Ethical Principles of Psychologists, in psychological studies had applied. An informed consent was obtained from all participants. All procedures performed in this research were following the institutional review board and national ethical

standards and according to the Helsinki settlement. This work does not include any clinical therapeutic trial.

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