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A novel mtDNA deletions are associated with diminished fertility in Iraqi human sperm.

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Abstract

Relationships between the genetic factors including sperm mtDNA mutations and diminished fertility and motility of human spermatozoa was evaluated in 30 fertile donors who had normospermic semen characteristics and from 50 subfertile or infertile patients. The latter included 23 with oligospermia, and 27 with asthenospermia males. Using long-range polymerase chain reaction (PCR), we confirmed the 4977 bp , 7345 bp and 7599 bp deletion of mtDNA in the spermatozoa with poor motility. The results showed that the ratio of the deleted mtDNA in the spermatozoa with poor motility and diminished fertility were significantly higher than those in the spermatozoa with good motility and fertility. In addition, we found that the frequencies of the three large-scale deletions in the spermatozoa from patients with asthenospermia and oligospermia were higher than those of the fertile males. Our findings suggest that mtDNA deletions may play an important role in some pathophysiological conditions of human spermatozoa. Mitochondrial genes ATPase6, Cytb, and ND1, were amplified by PCR and then analyzed by direct sequencing. However, a total of 75 different nucleotide substitutions were identified in the examined mitochondrial genes in both groups, all of which are statistically non-significant. sixteen substitutions caused an amino acid change and 37 were considered novel mutations. As a conclusion, mtDNA mutations in male infertility should be examined in larger numbers in order to clarify the effect of genetic disorders.

INTRODUCTION

Male infertility is a common condition affecting up to 54% of infertility cases, which comprise 10-14% of couples (Moore FL et al., 2000). One of the major factors associated with male infertility is the quality and quantity of sperm produced and sperm function such as sperm motility(Selvi RD et al .,2006). The conventional causes of male infertility are varicocele, cystic fibrosis, trauma, tumors, and genetic factors such as chromosomal abnormalities, Y chromosome microdeletions, single gene mutations, and sperm mitochondrial DNA (mtDNA) rearrangements **(**Kao SH et al., 1998;Agarwal A et al., 2008). The mutations occurring in mtDNA attract great interest in terms of some human diseases, either independent of or in association with nuclear DNA mutations **(**Taylor RW et al 2005). These alterations especially affect organs that have a high demand for respiratory energy. Because the mtDNA genes encode polypeptides for only the oxidative phosphorylation elements and enzymes (OXPHOS), mtDNA damages cause deficiency in ATP production (Solano A et al.,2001).

The mature human sperm contains 70-80 mitochondria located in the midpiece segment. Sperm mitochondria contain typical mtDNA, which is synthesized during meiosis and spermatogenesis. Furthermore, the mitochondrial genome remains active and is expressed in the ejaculated spermatozoa **(**Manfredi, G et al., 1997). Human mtDNA is a 16569 bp double stranded circular DNA molecule that codes for two rRNAs, 22 tRNAs, and 13 polypeptides that are essential for mitochondrial respiration and oxidative phosphorylation. Current concepts of the mitochondrial genetics embody five main features: (i) maternal inheritance, (ii) replicative segregation, (iii) high mutation rate, (iv) threshold in phenotypic expression of the mutant mtDNA, and (v) accumulation of somatic mtDNA mutations in ageing and degenerative diseases. Human mtDNA is compact (intron-less) and lacks the protection of histones or

DNA-binding proteins **(**Wallace DC et al., 1992). It replicates rapidly without efficient proof-reading and DNA repair mechanisms **(**Playán A et al., 2001), and thus has a mutation rate 10–100 times higher than that of nuclear DNA The flagellar propulsion of the sperm requires a large source of energy supplied by the mitochondria (Kao SH et al., 1998; Alcivar AA et al .,1989). Mitochondria play a key role in energy metabolism by containing OXPHOS .Since sperm movement requires a large amount of ATP to propel the flagellar apparatus, a defect in mitochondrial respiratory function will cause a decline in motility and fertility. Each mitochondrion has 2-10 DNA molecules (mtDNA) responsible for coding the few subunits of the OXPHOS enzymes. The ND1-ND6 and the ND4L genes encode seven complex I subunits (NADH-ubiquinone oxidoreductase); the Cytb gene encodes one complex III subunit (ubiquinol-cytochrome c oxidase oxidoreductase), and the ATPase6 and ATPase8 genes encode complex V subunits (ATP synthase) **(**Thyagarajan et al., 1997). mtDNA mutations affecting flagellar movement are a cause of sperm dysmotility. DNA rearrangements including point mutations and deletions of mtDNA have been reported in patients with low sperm motility who have asthenozoospermia and oligozoospermia (Kao SH et al., 1998;Folgero T et al., 1993; Lestienne P et al .,1997; Güney et al., 2012) As the severity of the spermatogenesis increases, the frequency of the microdeletions also increases. Indeed, we have demonstrated the association of the 4977 bp 7345 and 7599 bp deletion of mtDNA with diminished fertility and motility of human spermatozoa **(**Kao et al., 1995). In this study, we further analyzed sperm mtDNA deletions by long-range polymerase chain reaction (PCR) techniques and using the primer-shift PCR technique to know the association between sperm mtDNA deletions with low motility. The role that multiple mtDNA deletions may play in the pathophysiology of spermatozoa is discussed. To investigate the etiology of decreased fertility and motility of sperm and to develop an appropriate therapeutic strategy, the molecular basis of these defects must be ascertained. Therefore, we aimed to reveal the relationships between genetic factors including sperm mtDNA mutations in the ATPase6, Cytb and ND1 gene regions, large-scale deletions of mtDNAs in human spermatozoa and sperm parameters that could be regarded as candidate factors for male infertility in the Iraqi population.

Material and methods

Preparation and characterization of human spermatozoa

Fifty infertile men were recruited to the study. Of them, 27 asthenospermic subjects and the rest were oligospermic, according to the semen analysis results implemented Al-Karama and Al-Zahra Teaching Hospitals in Kut city/Iraq during the period October 2012 to June 2013. The control group consisted of 30 fertile and normospermic men who fathered at least one child. Seminal fluid from all subject enrolled in this study was obtained by masturbation after three-five days of sexual abstinence. The samples were collected in sterile, wide mouthed, non-toxic container and processed in the laboratory within an hour of ejaculation. All the semen samples underwent semen analysis in accordance with the WHO laboratory manual for the analysis of human seminal fluid and the study was performed using these reference values (WHO et al., 2010). Men were distributed into three groups according to the results of semen analysis (oligo, astheno and normospermic).

Sperm DNA extraction

Spermatozoa were collected and washed three times with five volumes of PBS (phosphate buffer saline; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) to avoid contamination from other cells such as lymphocytes and epithelial cells (Miller D et al.,1994).Total DNA of human spermatozoa was extracted (oligospermia, asthenospermia and controls) by using gSYNC™ DNA Extraction Kit which is designed for manual isolation of genomic DNA from fresh, frozen human blood according to the manufacturer's recommendations was performed,(May-Panloup P et al., 2003) except for the addition of Dithiothreitol (1% DTT) in order to break the sperm nucleus disulphide bonds, and also to dissociate mitochondria from the mitochondria sheath.

To amplify ND1, Cytb and ATPase6 mitochondrial genes, three sets of PCR primers were designed, which were located in the flanking regions of each gene and nine oligonucleotide primers pair used for the analysis of the 4977, 7345 and 7599 bp deletions in the mitochondrial DNA (mtDNAs) of human spermatozoa. Primer Design were based on the human mitochondrial sequence chemically synthesized by Pioneer oligo synthesis (Korea) . Lyophilized primers were dissolved in a free DNase/RNase water to give a final concentration of (100 pmol/µl) (as stock solution). The sequences of these primers were explained in all primer sequences were obtained from published literature as shown in table 1.

Polymerase chain reaction

The ND1, ATPase6 and Cytb genes were amplified in a total volume of 50 µl. PCR (Agilent Technologies Germany))was carried out in 0.2 ml PCR Microfuge tubes in a 25 µl reaction volume containing: 3 µl template genomic DNA (100-200 ng),12.5 µl PCR Master mix or Go Taq® Green Master Mix (Promega Madison, I,USA),

1.5 µl (2 µmol) each primer, and nuclease free sterile distilled water to 25 µl. The conditions of PCR amplification were as follows: a denaturation step at 94°C for 5 min followed by 35 cycles at 94°C for 1 min, 59°C for 1 min, and 72°C for 1 min, and then a final extension at 72°C for 5 min and stop at 4°C (Chen et al., 2004). The sizes of the fragments obtained after amplification were 934, 675 and 1064 bp for the ND1, Cytb and ATPase6 genes, respectively as shown in table 2. All PCR products were fractionated by run electrophoresis on 2% agarose gel .

Table 1: Primers used for PCR and the length of amplicons (Güney et al., 2012)

mtDNA gene	primer sequences (5'→3') of PCR	(nucleotide position)	Product Size(bp)
ND1	5'-primer: 5'-CCA ACC TCC TAC TCC TCA TTG T-3'	(3318-3339)	934
	3'-primer: 5'-GGG AAT GCT GGA GAT TGT AAT G-3'	(4231-4252)	
ATPase6	5'-primer: 5'-AAC GAA AAT CTG TTC GCT TCA T-3'	(8531-8552)	675
	3'-primer: 5'-ATG TGT TGT CGT GCA GGT AGA G-3'	(9185-9206)	
Cytb	5'-primer: 5'-ACC CCA ATA CGC AAA ATT AAC C-3'	(14751-4772)	1064
	3'-primer: 5'-TAC GGA TGC TAC TTG TCC AAT G-3'	(15794-5815)	

Primer-shift PCR.

In order to avoid artifacts in the detection of mtDNAs deletions, a primer-shift PCR method was employed to ascertain that the amplified DNA fragment was not due to misannealing of primers to the DNA template (Lee et al 1994). By using primer pairs L5–H4, L6–H6,and L6–H5 (Table 2), we could obtain PCR products of 555, 354,and 295 bp respectively; and with primers L2–H3, L3–H5, and L4–H3, we obtained 756, 702, and 406 bp PCR products respectively. This allowed us to confirm the presence of the 7345 and 7599 bp mtDNAs deletions respectively, in spermatozoa with poor motility. mtDNA was amplified from 15–20 ng of DNA in a 25 ml reaction mixture containing Green Master Mix. PCR was carried out for 35 cycles in a DNA thermal cycler (Agilent Technologies Germany) using the thermal profile of denaturation step at 94°C for 5 min followed by 35 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and then a final extension at 72°C for 5 min and stop at 4°C.

Long-range PCR.

A desired large segment (.8 kb) of mtDNA was amplified from 100 ng of sperm DNA .The Long Range PCR system (KAPA) is a blend of Taq DNA polymerase and a modified archaeal (Type B) DNA polymerase possessing proofreading capability. This two-enzyme system is designed specifically to support long range and/or sensitive C.The Long Range system polymerizes DNA from a primer annealed to a DNA template in the presence of deoxyribonucleotide triphosphate. Both enzymes possess 5-3’ polymerase activity, but only Taq possesses double strand dependent 5-3’exonuclease activity and only the Type B polymerase possesses 3-5’ exonuclease (proofreading) activity. PCR was carried out for 35 cycles using the thermal profile of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and primer extension at 72°C for 8 min. One more cycle of 20 s extension at 72°C was added for each of the last 15 cycles. The long-range PCR products were then separated on a 1.5% agarose/synergic gel at 150 V for 1.5 h and stained with 0.5 mg/ml ethidium bromide.

DNA Sequencing

Automated DNA sequencing was performed using sequenced with the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA). The primers used for PCR and direct sequencing of the ND1, ATPase6 and Cytb genes are shown in Table 3. All the data obtained from automated sequence was edited with a computer based program Sequencer. These sequences were compared with the reference sequence (NCBI database, accession number:NC-012920.1) to determine the nature of mutations.

RESULTS

The results revealed four major bands (with lengths of ~8360, 3383, 1015, and 761 bp, respectively) on the gel after ethidium bromide staining as shown in Figure 1. The 8360 bp band was the full-length PCR product from the wild type mtDNAs, the 3383 bp band was generated from the 4977 bp-deleted mtDNAs, the 1015 bp band was obtained from the 7345 bp-deleted mtDNAs, and the 761 bp band was amplified from the 7599 bp-deleted mtDNAs.. The 4977, 7345, and the 7599 bp deletions of mtDNAs were found more frequently in the spermatozoa with low motility. Spermatozoa in lanes 1–5 except Lane 4 are the blank, in which the sperm DNA was omitted from the reaction mixture. Lane M is the DNA size marker KAPA Universal DNA Ladder(100 ng/µl).

Using the primer-shift PCR technique according to the scheme noticed in figure 2 which illustrating the strategy used for the confirmation of large-scale deletions of mtDNAs by the primer-shift polymerase chain reaction (PCR) method, we obtained the PCR products with sizes in agreement with the shift in the distance of different primer pairs .By using the primer pairs L6–H5 (lane 4), L6–H6 (lane 3), and L5–H4 (lane 2), we obtained the PCR products of 295, 354, and 555 bp respectively, from 7345 bp-deleted mtDNAs as shown in figure 2. Similarly, we obtained the PCR products of 406 and 702 bp from the 7599 bp-deleted mtDNAs using primer sets of L4–H3 (lane 4) and L3–H5 (lane 6) respectively and nothingness the PCR products by using primer L2–H3 (lane 1) respectively. Lane 7 is the blank in which the PCR was carried out with all the reagents except the sperm DNA. DNA size marker KAPA Universal DNA Ladder (100 ng/µl).

Table 2. Oligonucleotide primers used for the analysis of the 4977, 7345 and 7599 bp deletions in the mitochondrial DNA (mtDNAs) of human spermatozoa(Kao et al., 1998**).**

Primer pair	Amplified position (nucleotide position)	Length of PCR product amplified from normal mtDNA (bp)	Length of PCR product amplified from deleted mtDNA (bp)
L1– H1a	3304-3717	414	414
L1–H2a	3304-3836	533	533
L2–H3d	7901-16255	8355	756
L3–H5d	8150-16450	8301	702
L4–H3d	8251-16255	8005	406
L5–H4c	8531-16430	7900	555
L6–H6c	8811-16509	7699	354
L6–H5c	8811-16405	7640	295
L3–H6e	8150-16509	8360	3383b, 1015c, 761d

a =The primer sets used for the determination of the total mtDNAs.
b =The primer sets used for the determination of the 4977 bp-deleted mtDNAs.
c =The primer sets used for the determination of the 7345 bp-deleted mtDNAs.
d =The primer sets used for the determination of the 7599 bp-deleted mtDNAs.
e =The primer sets used for long-range polymerase chain reaction (PCR).
L1 (3304–-3323) 59-AACATACCCATGGCCAACCT-39
L2 (7901–7920) 59-TGAACCTACGAGTACACCGA-39
L3 (8150–8166) 59-CCGGGGGTATACTACGGTCA-39
L4 (8251–8270) 59-GCCCGTATTTACCCTATAGC-39
L5 (8531–8550) 59-ACGAAAATCTGTTCGCTTCA-39
L6 (8811–8830) 59-CACCCAACTATCTATAAACC-39
H1 (3717–3698) 59-GGCTACTCGTCGCAGTGCGC-39
H2 (3836–3817) 59-GGCAGGAGTAATCAGAGGTG-39
H3 (16255–16236) 59-CTTTGGAGTTGCAGTTGATG-39
H4 (16430–16411) 59-TGCGGGATATTGATTTCACG-39
H5 (16450–16431) 59-CGAGGAGAGTAGCACTCTTG-39
H6 (16509–16490) 59-AGGAACCAGATGTCGGATAC-39

Table 3: The primer sequences used for PCR and direct sequencing of human mtDNA genes.

mtDNA gene	primer sequences (5'→3') of PCR
ND1	5'-primer: 5'-CCA ACC TCC TAC TCC TCA TTG T-3'
	3'-primer: 5'-GGG AAT GCT GGA GAT TGT AAT G-3'
ATPase6	5'-primer: 5'-AAC GAA AAT CTG TTC GCT TCA T-3'
	3'-primer: 5'-ATG TGT TGT CGT GCA GGT AGA G-3'
Cytb	5'-primer: 5'-ACC CCA ATA CGC AAA ATT AAC C-3'
	3'-primer: 5'-TAC GGA TGC TAC TTG TCC AAT G-3'

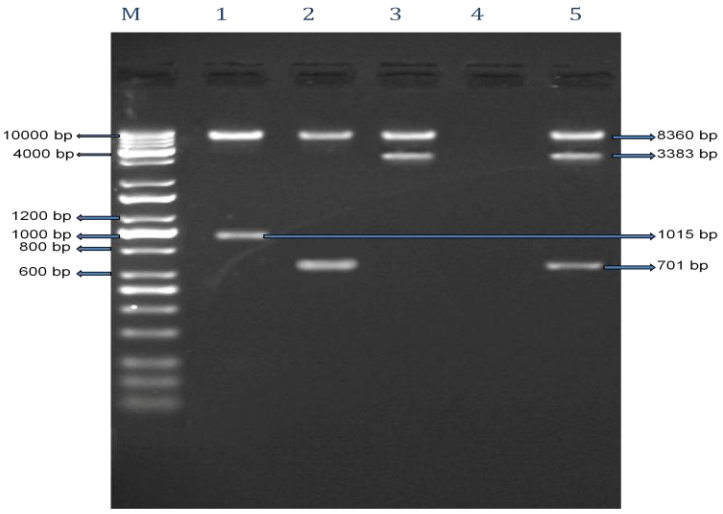

Figure 1. Demonstration of large-scale deletions of mtDNAs in infertile male patients by long-range polymerase chain reaction (PCR).

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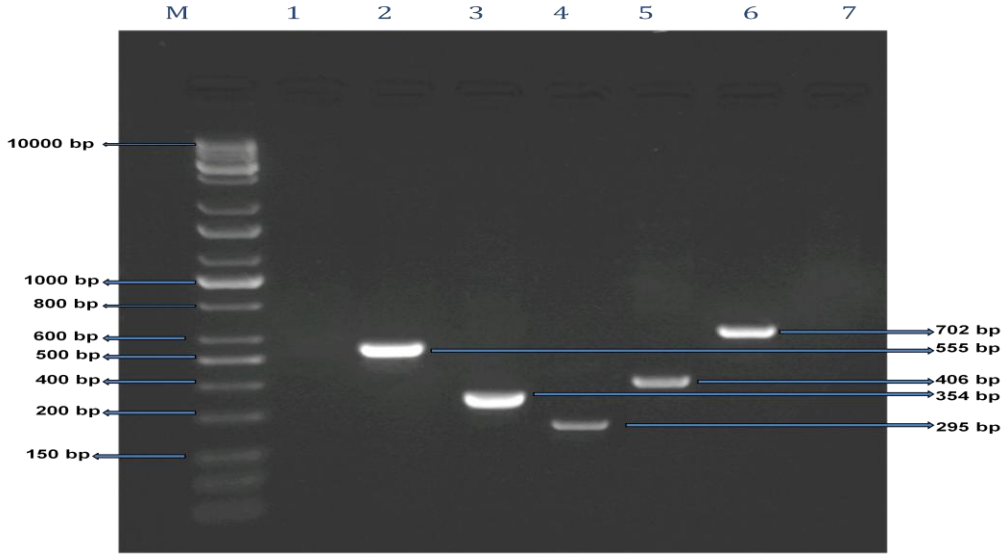

Figure 2. Detection of mtDNAs molecules with the 7345 and 7599 bp deletions by the primer-shift polymerase chain reaction (PCR) method

Occurrence frequency of the 7345 , 7599 and 4977 bp deletions of mtDNAs in human spermatozoa

The frequency of occurrence of the 7345, 7599 and 4977 bp-deleted mtDNAs in the spermatozoa with different motility and fertility was observed. Higher incidence was found in the spermatozoa with poor motility and diminished fertility compared with the spermatozoa with good motility and fertility (Table 3). The frequencies of occurrence of the 7345 bp-deleted mtDNA were 10%(L5-H4), 26%(L6-H6), 6%(L6-H5) and 8%(L3-H6) for the spermatozoa. The average incidences of the 7599 bp-deleted mtDNA were 10%(L3-H5), 16%(L4-H3), and 4%(L3-H6) for spermatozoa. The frequencies of occurrence of the 4977 bp-deleted mtDNA were 4%(L3-H6). The frequencies of occurrence of the 7345,7599 and 4977 bp deleted mtDNA were 50%,30% and 4/98+% respectively (the number of subjects with the indicated deletion/total number of subjects in each tested group) for the study subjects with normal fertility and males with asthenospermia, and oligozoospermia respectively. These data suggested a higher incidence of mtDNA deletions in the male with asthenozoospermia, and oligozoospermia. It is

noteworthy that no significant correlation was found between the frequency of occurrence of the large-scale mitochondrial DNA deletions and the age of all the study groups(normo ,oligo and asthenospermic males) .

The large-scale deletions of mtDNAs

These three large-scale deletions (4977, 7345- and 7599 bp) were found to occur either alone or in different combinations. Among them, the 4977 bp deletion was the most frequently seen in spermatozoa with poor motility. in addition to farther deletion 7345- and 7599 bp-deleted mtDNAs in the spermatozoa from infertile males. Analysis of the nucleotide sequences found the seven nucleotide (59-CATCAAC-39) is located in the np 8643-8637(ATPase6) was high frequent of mutant in the male with asthenozoospermia, and oligozoospermia as observed in Fig(3). Analysis of the human mtDNAs revealed five CATCAAC direct repeats, which include np 5617–5611, np 8643–8637, np 13296–13290, np 16079–16073, and np 16242–16236 on the heavy strand Except for np 5617–5611(Agarwal A et al., 2008) The nucleotide sequences flanking the breakpoints of the 7599 bp deletion revealed a seven nucleotide direct repeat, which is generally found in the large-scale deletions of mtDNAs and that data agree well with that reported by (Agarwal et al., 2008**)** and (Holt I et al .,1994). The nucleotide sequences within these regions were found to assume unusual structures, such as bent DNA and anti-bent DNA as reported by(Seitz et al., 1995, Hou;, J.H et al., 1996). These regions may be thus more vulnerable to attack by free radicals or serve as recognition motifs for certain recombination machinery involved in the large-scale deletions. .

Table 3: Oligonucleotide primers used for the analysis of the 4977, 7345 and 7599 bp deletions in the mitochondrial DNA (mtDNAs) of human spermatozoa. O*=oligospermic

A*= asthenospermic

	Primer pair	Length of PCR product amplified from deleted mtDNAs (bp)	multiple mtDNAs deletions of human spermatozoa in 10 controls	multiple mtDNAs deletions of human spermatozoa included 17 male with oligozoospermia, and 23 with asthenospermia
3	L2–H3	756(7599 bp dmtDNA)	0/10	0/40
4	L3–H5	702(7599 bp dmtDNA)	0/10	2/17and 3/23(10%)
5	L4–H3	406(7599 bp dmtDNA)	2/10	1/17 and5/23(16%)
6	L5–H4	555 (7345 bp dmtDNA)	1/10	4/23(10%)
7	L6–H6	354(7345 bp dmtDNA)	1/10	4/17 and 8/23(26%)
8	L6–H5	295(7345 bp dmtDNA)	0/10	1/17 and2/23(6%)
9	L3–H6	3383 (4977 bp dmtDNA)	0/10	1/17 and1/23(4%)
		1015(7345 bp dmtDNA)	1/10	3/23(8%)
		,761(7599 bp dmtDNA)	0/10	1/17 and1/23(4%)

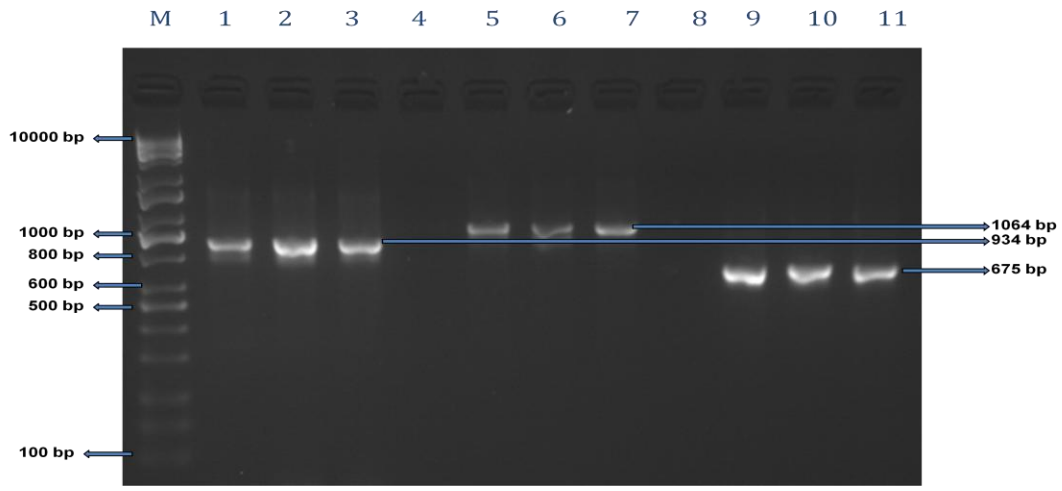

Figure 3 : The sizes of the fragments obtained after amplification were 934, 1064 and 675 bp for the ND1(lane1,2,3), ATPase6(lane5,6,7)and Cytb genes(lane 9,10,11), respectively and(lane 4,8) are the blank in which the PCR was carried out with all the reagents except the sperm DNA.All PCR products were fractionated by electrophoresis on 2% agarose gel. DNA size marker was KAPA Universal DNA Ladder (100 ng/µl).

Table 4. The mtDNAs mutations identified in the ATPase6, Cytb, and ND1 regions.

Nucleotide position	N.B change	Amino Acid change	Mutation in N*	Mutation in O*	Mutation in A*	Mutation frequency in cases	Reported in mt DNA map
				ATPase6 gene			
8635	C→A	L→I	**1**	**2**	**2**	**5/5**	X
8637	C→A	silent	**1**		**1**	**2/5**	Novel
8637	C-del	L-fs-F		**1**		1/5	Novel
8639	T→A	I→N	**1**	**1**	**2**	**4/5**	Novel
8640	C-del	I-fs-N			**1**	**1/5**	Novel
8655	C→T	silent			**1**	**1/5**	X
8701	A→G	T→A	**1**	**1**		2/5	X
8784	A→G	silent	**1**			1/5	X
8860	T→G	T→A	**1**	**2**	**2**	**5/5**	X
8877	T→C	silent	**1**			1/5	X
8959	G→A	silent			**1**	**1/5**	Novel
9072	A→G	silent	**1**			1/5	X
9193	C-ins	HDNTL-frame shift-pKKQM		**1**		1/5	Novel
9193	A-ins	HDNTL-frame shift-pKKQM		**1**		1/5	Novel
9195	C→A	L→Q		**2**	1	3/5	Novel
9196	G→A	D→N		**2**		2/5	X
9204	A→C	silent	**1**	**1**		2/5	X
9205	T→A	**Term**→Q		+		2/5	X

Cytb gene							
14791	C→T	silent	**1**			1/5	X
14798	T→C	F→L			**1**	**1/5**	X
14801	A-del	IDLPTPSN-fs-SRPPHPIH		**1**		1/5	Novel
14823	A-del	N-fs-H		**1**		1/5	Novel
14854	C→G	silent	**1**		**1**	**2/5**	Novel
14855	C→T	L→F	**1**	**1**	**1**	**3/5**	Novel
14860	C→G	silent	**1**	**1**	**1**	**3/5**	Novel
14911	C→T	silent	**1**			1/5	X
15115	T→G	silent			**1**	1/5	X
15148	G→A	silent			**1**	1/5	X
15216	G→T	G →V		**2**		**2/5**	Novel
15217	G→T	silent		**1**	**1**	**2/5**	X
15218	A→T	T→S		**1**	**1**	**2/5**	Novel
15326	A→G	T→A	**1**	**2**	**2**	**5/5**	X
15398	A-ins	ITFHPYLY TI-fs-NHLPLLHN Q			**1**	1/5	Novel
15427	A-ins	DA-fs-TP			**1**	1/5	Novel
15452	C→A	L→I		**1**	**1**	2/5	X
15499	C→G	silent	**1**			1/5	X
15500	G→C	D→L	**2**			2/5	X
15510	A-ins	NYTLANPL NT-fs-KLYYPSQP LKH			**1**	1/5	Novel
15575	G→A	A→T			**1**	1/5	Novel
ND1gene							
3337	G→A	V→M	**1**	**1**	**2**	4/5	X
3340	C→T	P→S		**1**	**1**	2/5	X
3342	C→A	silent		**1**		1/5	X
3368	T→A	M→K			**1**	1/5	Novel
3376	G→A	E→K			**1**	1/5	X
3381	A→G	silent		**1**	**1**	2/5	X
3414	C→T	G→A			**1**	1/5	Novel
3417	C→A	N→Q	**1**		**1**	2/5	Novel
3420	C-del	NVV-fs-QVG	1	1	**1**	3/5	Novel
3421	CG-del	N-fs-S		**1**	1	2/5	Novel
3423	T→G	silent		1	**2**	**3/5**	X
3425	T→G	V→A	**1**	1		2/5	X
3426	A→C	silent			1	1/5	X
3429	C→G	silent			**1**	1/5	X
3441	A→T	silent	**1**			1/5	X
3460	G→A	A→T	**1**			1/5	X
3464	T→A	M→ H		1	**2**	**3/5**	Novel
3470	T→A	L→H			**1**	1/5	Novel
3476	C→A	T→N	**1**	**1**		2/5	Novel
3479	A→C	K→T		**1**	**1**	2/5	Novel
3483	G→T	E→V		1		1/5	Novel
3516	C→G	silent	**1**	1	**1**	**3/5**	X

3543	C→T	silent			**1**	1/5	X
3552	T→G	silent		**2**	**2**	4/5	X
3594	C→T	silent		**2**		2/5	X
3607	G→T	G→C			**1**	1/5	Novel
3623	T→C	L→P		**1**		1/5	Novel
3625	C→G	A→G		**2**		2/5	Novel
3639	A→G	silent	**1**			1/5	Novel
3644	T→G	V→G			**2**	2/5	X
3782	C→T	S→F			**1**	1/5	Novel
3787	A→T	N→Y		**2**		2/5	Novel
3810	A→G	silent	1	2		3/5	X
4083	T-ins	VTKTLLLT SLFLWIRT AYPRFRYD QLMHLW-fs-CHQDPTSN LPVLMNSN MPPIPLRPT HTPPM			**1**		Novel
4181	A-del	NFLPLT-fs-TSYHSP			**1**	1/5	Novel
4216	T→C	Y→H		**1**	**1**	2/5	X

N*=Normal A*= Asthenospermia O*= Oligozoospermia

All polymorphism found in the sequenced mtDNAs regions are summarized in Table 4. Briefly, 75 different nucleotide substitutions were identified, of which 16 caused an amino acid change. In addition, 37 were considered novel mutations which five of them are silent mutation(don't caused an amino acid change) and the other thirty two caused an amino acid change.T8639A, C9195A, deletion(C) np 8673,8640 and insertion(A,C) np 9195 novel polymorphisms were found in the ATPase6 gene, and C14801T, G15216T, A15218T, G15575A, deletion(A) np 14801,14823 and insertion(A) 15398,15427 and 15510 novel polymorphisms were found in the Cytb gene. Also C3414T, C3417A, T3464A , T3470A,C3476A,,C3425G,C3782T , A3479,G3483T ,G3483T,G3607T,T3623C, A3787T ,deletion(C,CG)np 3420,3421 and insertion(T,A)np 4083 and 4181 novel polymorphisms were found in the ND1 gene.

Discussion

Male infertility accounts for half of the etiology of infertility in couples. Infertility itself affects about 10-14% of all couples attempting pregnancy. The suspected causes of male infertility include sperm motility disorders ,Y chromosome microdeletions, chromosomal abnormalities, hormonal and/or receptor disorders (Güney et al .,2012). Mitochondrial DNA mutations affecting flagellar movement is also one of the causes of sperm dysmotility. DNA rearrangements including point mutations and deletions of mtDNA have been reported in patients with low sperm motility who have asthenozoospermia and oligoasthenozoospermia (Kao SH et al .,2003; Folgero T et al .,1993; Lestienne P et al .,1997). Therefore, in this study, the effects of mtDNA mutations on male infertility were examined.

The major determinant of sperm motility is strongly dependent on ATP biosynthesis, which is carried out by the mitochondrial OXPHOS (Ruiz-Pesini et al., 1998) . The mutation rate of mtDNA is 10-20 times higher than that of nuclear DNA (Wallace, 1992). Lack of histone protection and efficient proofreading mechanism, high turnover rate, and exposure to ROS are the main reasons of high mtDNA mutation rates(Kumar et al 2009). As the mitochondrial genome does not contain intron, any mutation occurring in mtDNA would affect the coding regions. Therefore, a variety of neutral and deleterious mutations are found in human populations In recent years, the effect of mtDNA mutations on male infertility has been studied. The mtDNA defects, which include point mutations or multiple deletions of mtDNA, have been found in patients with asthenozoospermia(Folgero et al 1993; oligozoospermia (Lestienne et al., 1997). In addition, in some studies, deletions in mtDNA tend to develop a decline in motility and fertility in human sperm(Kao et al 1998; Cummins (1998).; Cummins et al., 1998; O'Connell et al., 2002). Furthermore, it has been demonstrated that mtDNA base substitutions can greatly influence semen quality (Chao et al., 2004). Therefore, we investigated the relationship between the mtDNA genes ATPase6, Cytb and ND1 and male

infertility. In the present study, the possible role of an mtDNAs mutation in the pathophysiology of human sperm dysfunction was investigated to develop an appropriate therapeutic strategy; the molecular basis of these defects must be elucidated. We have investigated large-scale deletions of sperm mtDNAs in 50 subfertile and infertile males by using long-range PCR techniques. In addition to the previously reported common 4977 bp deletion, we found two other deletions of the 7345 and 7599 bp of mtDNAs in the spermatozoa with low motility scores (Figure 3), and novel 37 mutation in Iraqi population as shown in table (3).These three large-scale deletions were found to occur either alone or in different combinations. Among them, the 4977 bp deletion was the most frequently seen in spermatozoa with poor motility. While the 4977 bp deletion was also present in the somatic tissues, we could only detect the 7345- and 7599 bp-deleted mtDNAs in the spermatozoa from infertile or subfertile males. The mechanisms by which these large-scale mtDNAs deletions occur in mitochondria remain unclear. In the past decade, several mechanisms have been proposed, including slipped-impairing (Shoffner et al.,1989), illegitimate recombination (Mita et al., 1990), oxidative reactions elicited by free radicals, and DNA strand break effected by a topoisomerases or DNA recombinase (Poulton, et al .,1993).Analysis of the nucleotide sequences flanking the breakpoints of the 7599 bp deletion revealed a seven nucleotide direct repeat, which is generally found in the large-scale deletions of mtDNAs and that data agreed well with that reported by Johns,et al (1989). The seven nucleotide (59-CATCAAC-39) is located in the np 8643-8637(ATPase6) was high frequent of mutant in the male with asthenozoospermia, and oligozoospermia (Figure 4). These regions may be thus more vulnerable to attack by free radicals or serve as recognition motifs for certain recombination machinery involved in the large-scale deletions (Mita et al., 1990) .Thus, some other factor(s) may be involved in eliciting the large-scale deletions of mtDNAs. In a previous study by Kao et al., (1998),they demonstrated that the 4977 bp deletion of mtDNAs also occurs in the spermatozoa of infertile and subfertile males. The 4977 bp deletion of mtDNAs causes the removal or truncation of multiple structural genes (ATPase 6/8, COIII, ND3, ND4L, and ND4) and five tRNA genes. The 7345 and 7599 bp deletions cause a loss or truncation of the structural genes of ATPase 6/8, COIII, ND3, ND4L, ND4, ND5, ND6, Cytb, and eight tRNA genes. These mtDNAs deletions may result in multiple respiratory chain deficiencies (Lee and Wei, 1997).Defective respiratory enzymes containing protein subunits encoded by the deleted mtDNAs may further enhance free radical production, resulting in more profound oxidative damage. Spermatozoa are especially liable to oxidative damage because their plasma membranes are rich in poly-unsaturated fatty acids. Abnormalities in the ultrastrucural of sperm midpiece were found to increase in the spermatozoa treated with lipoperoxides (Rao et al., 1989). It has been established that mitochondrial dysfunction may be caused by mtDNAs mutation and oxidative damage caused by endogenous and exogenous free radicals (Yakes, F.M. and van Houton, B. (1997). As reactive oxygen species (ROS) are continuously generated by the respiratory chain, they may cause significant oxidative damage to mtDNAs if not efficiently eliminated. Fraga et al., (1997) reported that smoking, which depletes antioxidants and increases oxidative stress, induced a significant increase of 8-hydroxy-29-deoxyguanosine in sperm DNA. In the presence of mtDNAs mutations (especially multiple deletions), which may be caused by ROS or free radicals generated during normal aerobic metabolism, the spermatozoa are deprived of ATP (due to the defective mitochondria respiratory functions and will then run into a state of 'energy crisis' through a 'vicious cycle' as recently proposed by Wei (1998). This 'vicious cycle' may have catastrophic consequences and is accelerated by electron leakage from defective mitochondria; as such, it may play an important role in the pathophysiology of some male infertility and subfertility. On the other hand, (Kitagawa et al (1993) reported that the 4977 bp-deleted mtDNAs is accumulated in post-menopausal ovaries. The 4977 bp mtDNAs deletion was detected in the oocytes of women aged 45 years (Chen et al .,1995). The mutations in the mtDNAs of germ cells are possibly generated by a change of environment of the germ cells (e.g. hormone, oxygen pressure, and generation of reactive oxygen species during human ageing.

These observations are compatible with the reduced sperm output, increased epididymal transit time, greater number of Sertoli cell abnormalities, and sporadic spermatogenetic failure that occurs in the reproductive systems of elderly males (Chen et al., 1995). During the process of spermatogenesis, the mitochondria of male germ cells undergo dramatic segregation and morphological changes(Johnson 1988), in which mtDNAs mutations may be introduced. The mtDNAs mutations may occur and accumulate in the original spermatids or during spermatogenesis and thereby impair respiratory function and motility
of spermatozoa.

Previously, multiple deletions of mtDNAs were revealed in a patient with oligoasthenozoospermia by Lestienne et al(1997).They proposed that the multiple mtDNAs deletions may be of nuclear origin since three nuclear loci have been ascribed to multiple mtDNAs deletions in the human. A variation of a nuclear gene product affecting the integrity of mtDNAs, and thus sperm motility, might be transmitted to the offspring of infertile males undergoing intracytoplasmic sperm injection (ICSI). This consideration is substantiated by our findings that the 4977, 7345, and 7599 bp deletions of mtDNAs are presenting spermatozoa with declined fertility and motility. Thus, it is advisable to

perform analysis of mtDNAs mutations in spermatozoa from infertile or subfertile males who are about to participate in the ICSI programme. It remains unknown whether the three large-scale deletions are caused by nuclear factors. The mtDNAs mutations in the spermatozoa might have occurred in the oocytes of the mothers of the infertile males examined in this study.

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