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Original Article

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Correlation of Sperm Mitochondrial DNA 7345 *bp* and 7599 *bp* Deletions with Asthenozoospermia in Jordanian Population

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Abstract

Background: Alterations in sperm mitochondrial DNA (mtDNA) affect the functions of some OXPHOS proteins which will affect sperm motility and may be associated with asthenozoospermia. The purpose of this study was to investigate the correlation between 7599-*bp* and 7345-*bp* sperm mtDNA deletions and asthenozoospermia in Jordan.

Methods: Semen specimens from 200 men including 121 infertile and 79 healthy individuals were collected at the Royal Jordanian Medical Services In-vitro fertilization (IVF) units. The mtDNA was extracted followed by mtDNA amplification. Polymerase chain reaction (PCR) was conducted for the target sequences, then DNA sequencing was performed for the PCR products. Chi-square, Fisher's and Spearman's tests were used to calculate the correlation.

Results: The results showed a significant correlation between the presence of 7599-*bp* mtDNA deletion and infertility where the frequency of the 7599-*bp* deletion was 63.6% in the infertile group compared to the fertile 34.2% ($p<0.001$, (OR=3.37, 95% CI=1.860 to 6.108)). Additionally, the sperm motility showed a significant association with the frequency of the 7599-*bp* deletion ($p=0.001$, $r=-0.887$). The 7345-*bp* mtDNA deletion showed no asoctiation with the infertility ($p=0.65$, (OR=0.837, 95% CI= 0.464-1.51)) or asthenozoospermia ($p=0.98$, $r=0.008$).

Conclusion: We demonstrated a significant correlation between asthenozoospermia and the 7599-*bp* mtDNA deletion but not the 7345-*bp* mtDNA deletion in the infertile men in Jordan. Screening for deletions in sperm mtDNA can be used as a predignostic molecular marker for male infertility.

Keywords: Asthenozoospermia, Gene deletion, Male infertility, Mitochondrial DNA, Sperm motility.
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Introduction

Infertility is the inability of a couple to conceive a child after one year of regular coitus without using contraception (1). It may be attributable to male or female factors (2). Approximately, 17% of couples are suffering from this condition (3). Among all infertility cases, 50% of cases are related to male factors (4).

The evaluation of the seminal fluid is generally based on the assessment of its volume, sperm-count, liquefaction time, sperm motility, and mor-

phology (5). Sperm motility is essential in the fertilization process because it makes the sperm able to migrate from the vagina towards the fallopian tube and penetrate the ovum (6). Reduction in the motility of sperm is called asthenozoospermia which is a cause of male infertility that is characterized by decreased sperm motility below 40% (7). The rapid forward progressive movement of sperm is relying on energy (ATP) supplied by sperm mitochondria via the oxidative phosphorylation (OXPHOS) process (8). The OXPHOS genes are located on the mitochondrial DNA (mtDNA) genome. In humans, the mtDNA genome is a circular double-stranded DNA molecule of 16569 *bp* that encodes 13 polypeptides, two ribosomal ribonucleic acids (rRNAs) and 22 transfer ribonucleic acids (tRNAs) that play an essential role in the mitochondrial respiration and oxidative phosphorylation (9). Moreover, mtDNA has a large number of direct repeats of different sizes ranging between 4 to 17 *bp* (10) (Figure 1). Human mtDNA, compared to nuclear DNA, has different and unique properties like fast replication and lack of an efficient proofreading mechanism. Therefore, the rate of mtDNA alterations is expected to be 17 times higher than nuclear DNA (11, 12). Lack of DNA repair mechanism makes the mtDNA more vulnerable to the attack of normally formed free radicals and reactive oxygen species (ROS) (13).

The most common mtDNA deletion is the 4977 *bp* deletion, which tends to affect 40% of individuals with mitochondrial dysfunctions (14). Some studies in different populations reported a correlation between the 4977 *bp* mtDNA deletion and asthenozoospermia (12), whereas, in some other populations, there was no correlation between the 4977 *bp* deletion and the abnormalities in count, morphology or, motility of spermatozoa (15) (Figure 2). Moreover, other mtDNA deletions showed an association with poor sperm motility; for instance, 7599 and 7345 *bp* deletions have been studied by Kao et al. (9) and they reported a significant correlation between these mtDNA deletions and poor sperm motility. However, Talebi et al. (2018) reported that the 7599 *bp* mtDNA deletion was associated with asthenozoospermia, and no correlation was detected between the 7345 *bp* mtDNA deletion and reduced sperm motility (16).

The mtDNA 7599 *bp* deletion is located between 8642 and 16243 *bp* and is characterized by the presence of seven-nucleotide direct repeats (5'-CATCAAC-3') at both sides whereas, 7345 *bp* mtDNA deletion lies between 9009 and 16354 *bp* (9). The molecular approaches have shown the association between 7599 and 7345 *bp* mtDNA deletion with functionality of some mitochondrial genes including ATPase8, ATPase 6, cytochrome c oxidase (COX) III, cytochrome b, NADH dehydrogenase (ND) 3, 4, 4L, 5, and 6. Also, those deletions include losing 8 tRNA genes (Figure 3) and all of these genes play a significant role in the

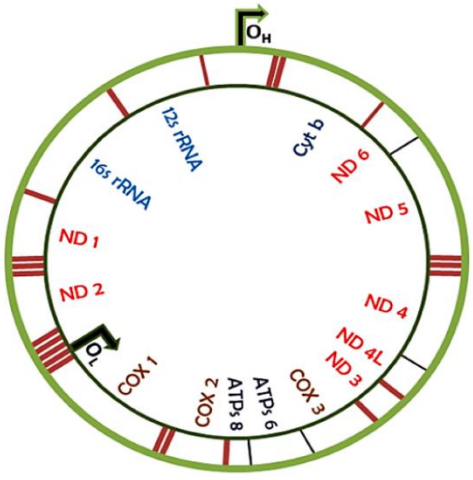

Figure 1. The human mitochondrial DNA (mtDNA). The genome consists of 37 genes coding for 13 polypeptides, 22 tRNAs, and 2 rRNAs (16S and 12S). The starting points of replication on the heavy strand (O_H) and the light strand (O_L) are indicated

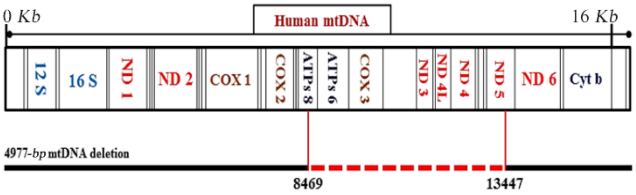

Figure 2. The 4977 *bp* (Common deletion) within the whole mtDNA. Deleted genes are indicated between the vertical red lines

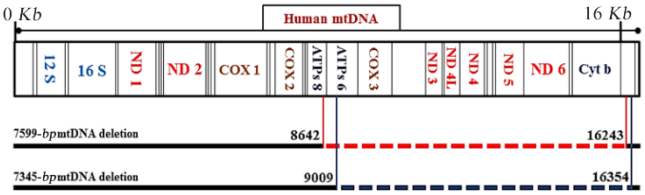

Figure 3. Locations and deleted genes within 7599 and 7345 *bp* mtDNA deletions. The sites of both deletions and the lost mtDNA genes appear; 7599 *bp* mtDNA deletion is between the two red lines, whereas 7345 *bp* mtDNA deletion is indicated within the blue lines

construction of mature sperm and progressive flagellar movement after ejaculation (16).

Asthenozoospermia is considered the main cause of male infertility (17). Thus, screening of human mitochondrial DNA for mutations can be helpful in the molecular diagnosis of male infertility. Therefore, in this study, the association of 7599 and 7345 *bp* human mitochondrial DNA deletions was investigated among Jordanian population.

Methods

Specimen collection and semen analysis: Semen specimens from 121 patients diagnosed with asthenozoospermia of ages between 18 and 40 years were collected between Jan 2017 and Dec 2017, and divided into six groups according to sperm motility; group one included 20 patients with 0% sperm motility (Completely immotile), group two included 20 patients with 1% to 5% sperm motility, group three involved 20 patients with 6% to 10% sperm motility, group four included 20 patients with 11% to 15% sperm motility, group five comprised 20 patients with 16% to 20% sperm motility, and finally, group six included 21 patients with 21% to 35% sperm motility.

Sample collection and all other protocols were approved by the local institutional review board at Yarmouk University. The consent form was signed by each participant after a verbal explanation of the objectives of the study.

Based on the exclusion criteria of the study, patients who drank alcohol and smoke cigarettes in addition to individuals who had a varicocele and aged more than 40 years were excluded from the study (10).

Semen specimens from 79 men with normozoospermia (High percentage of sperm motility, >50%) were collected which comprised the control infertile group. Similarly, samples were also subcategorized according to the percentage of sperm motility into three groups; group one included 25 samples with motility of 50% to 55%, group two involved 27 samples with sperm motility of 56% to 60%, and finally, group three included 27 samples with sperm motility of 61% to 75% (18).

Semen specimens were collected from in vitro fertilization (IVF) units of Jordanian Royal Medical Services at King Hussein Medical Center and Prince Rashid Ben Al-Hasan Military Hospital, where infertile patients provided their seminal fluid for evaluation. Seminal fluid specimens from all subjects were obtained by masturbation after three to five days of sexual abstinence. Semen specimens were collected in a special sterile, wide-mouth and non-toxic container and placed in the lab. Semen specimens were incubated at 37°*C* to allow liquefaction to occur for 30 *min*. Then, semen specimens were evaluated by a senior clinical embryologist following the guidelines of WHO laboratory manual for the examination and processing of human seminal fluid (19).

Semen specimens were fractionated using Percoll media (45% and 90% gradients) and centrifuged at 1000×*g* for 20 *min*. After that, the pellets were collected and washed two times with sperm washing medium to get rid of Percoll remnants, then spermatozoa were collected by centrifugation using microcentrifuge at 670×*g* for 10 *min*. Finally, the pellet containing spermatozoa was kept at −20°*C* until DNA extraction (20).

Sperm mtDNA amplification: Total DNA was extracted using QIAamp DNA Mini Kit (Qiagen, Germany) following the manufacturer's instructions. After that, the mitochondrial DNA of spermatozoa was amplified using REPLI-g Mitochondrial DNA Kit (Qiagen, Germany) following the manufacturer's recommendations. Then, the density ratio for amplified mitochondrial DNA from all specimens was measured, and specimens that had an optimal density ratio (260/280) close or equal to 1.8 were chosen and stored at −20°*C*.

Amplification of mtDNA: Polymerase chain reaction (PCR) was performed to confirm the occurrence of the mtDNA in the extracted genome reaction; for this purpose, the sample was prepared with a total volume of 30 *µl* containing 15 *µl* of 2X master mix (GoTaq, Promega, USA), 1 *µl* of each primer, 3 *µl* of diluted amplified mtDNA and 10 *µl* of nuclease-free water. The PCR was performed using specific primer pair (F1-R1) (Table 1), and the fragment size of the amplicon was expected to be 414 *bp* (Table 1). PCR was carried out using a thermal cycler (Bioer, China) upon the following program; the initial denaturation step was done at 95 ℃ for 2 *min* followed by 40 cycles of denaturation at 95 ℃ for 30 *s*, annealing step at 55 ℃ for 30 *s*, and extension at 72 ℃ for 1 *min*. Finally, the reaction was ended with the final extension step at 72 ℃ for 5 *min*.

The resulted PCR products were resolved by electrophoresis with 1% agarose gel at 100 *V* for 45 *min* using 1X Tris-Borate-EDTA (TBE) buffer. Next, they were visualized by UV and a 50-*bp* DNA marker was applied.

Table 1. Oligonucleotide primers used for mtDNA confirmation and PCR amplification of 7599 and 7345 *bp* deletions (Kao et al., 1998)

Primers	Primer sequences	Primer pairs	Amplified position	Amplicon size (*bp*)
F1	5'-AACATACCCATGGCCAACCT-3'	(F1-R1) mtDNA	3304–3717	414
R1	5'-GGCTACTGCTCGCAGTGCGC-3'			
F5	5'-ACGAAAATCTGTTCGCTTCA-3'	(F5-R4) 7345 *bp*	8531–16, 430	555
R4	5'-TGCGGGATATTGATTTCACG-3'			
F2	5'-TGAACCTACGAGTACACCGA-3'	(F2-R3) 7599 *bp*	7901–16, 255	756
R3	5'-CTTTGGAGTTGCAGTTGATG-3'			
F4	5'-GCCCGTATTTACCCTATAGC-3'	(F4-R3) 7599 *bp*	8251–16, 255	406
R3	5'-CTTTGGAGTTGCAGTTGATG-3'			

Detection of 7599 and 7345 bp mtDNA deletions: To detect the existence of 7599 and 7345 *bp* deletions in mtDNA samples, PCR reaction was performed using F4-R3, F2-R3, and F5-R4 primers pairs, respectively (Table 1), and the length of the PCR products was predicted to be 406, 756 *bp*, and 555 *bp*, respectively (Table 1). PCR amplification to detect both deletions was done and each sample in a total volume of 30 *µl* contained 15 *µl* of 2X master mix (GoTaq, Promega, USA), 1 *µl* of each one of the specific primers, 3 *µl* of diluted amplified mtDNA and 10 *µl* of nuclease-free water. PCR conditions for detection of 7599 *bp* deletion were started with initial denaturation step at 95 ℃ for 2 *min* followed by 35 cycles of denaturation at 95 ℃ for 30 *s*, annealing at 55 ℃ for 30 *s* and extension at 72 ℃ for 1 *min*. Then, the final extension step was performed at 72 ℃ for 5 *min*.

Next, PCR conditions to detect 7345-*bp* deletion were started with an initial denaturation step at 95 ℃ for 2 *min* followed by 40 cycles of denaturation at 95 ℃ for 30 *s*, annealing at 60 ℃ for 30 *s* followed by an extension step at 72 ℃ for 1 *min*. Finally, a final extension step was performed at 72 ℃ for 5 *min*.

The resulted PCR products were resolved by electrophoresis with 1% agarose gel at 100 *V* for 45 *min* using 1X Tris-Borate-EDTA (TBE) buffer. Next, they were visualized by UV and DNA marker of 50 *bp* was applied.

Statistical analysis: SPSS software was used for data analysis. Frequency was calculated for 7599 and 7345 *bp* mtDNA deletions among the study population. Spearman's correlation analysis was conducted to evaluate the relationship between the presence of the corresponding deletions and the reduction in sperm motility. Furthermore, Fisher's exact test was applied to calculate the odds ratio with 95% confidence intervals. The p-value was considered significant if it was ≤0.05. Graphs were drawn using GraphPad Prism 7.00 software.

Results

The study population consisted of 200 individuals including 121 infertile and 79 fertile men that were classified according to the sperm motility percentage into subgroups as shown in tables 2 and 3. There was no significant difference in the mean age between the infertile and fertile groups (p>0.05). The mean age of the infertile group was 34.4 years, whereas, the mean age for the control group was 32.7. The PCR amplification of the target sequences was confirmed by detection of the target bands on 2% agarose gel as shown in figure 4.

The results showed a significant difference in the presence of the 7599 *bp* mtDNA deletion in 77 infertile individuals (63.6%) compared to only 27 individuals (34.2%) of the control group, calculated by Fisher's exact test (p<0.001) (OR=3.37, 95% CI=1.860 to 6.108) (Table 2). However,

Table 2. Frequencies of 7599 and 7345 *bp* mtDNA deletions in infertile and fertile group

mtDNA deletion	Infertile		Fertile		p-value (odds ratio, 95%, CI)
	#	%	#	%	
7599 *bp*	77	63.6	27	34.2	<0.001 (OR=3.37, 95% CI=1.860 to 6.108)
7345 *bp*	41	33.9	30	38.0	0.650 (OR=0.837, 95% CI=0.464-1.51)

Table 3. Frequencies of 7599 *bp* mtDNA deletion in infertile and fertile group according to sperm motility

Motility	Number of cases	Presence of deletion	Percentage	p-value (spearman's correlation)
0%	20	14	70%	0.001 (r=-0.887)
1-5%	20	15	75%	
6-10%	20	13	65%	
11-15%	20	11	55%	
16-20%	20	14	70%	
21-35%	21	10	48%	
50-55%	25	13	52%	
56-60%	27	10	37%	
61-75%	27	4	15%	
Total	200	104		

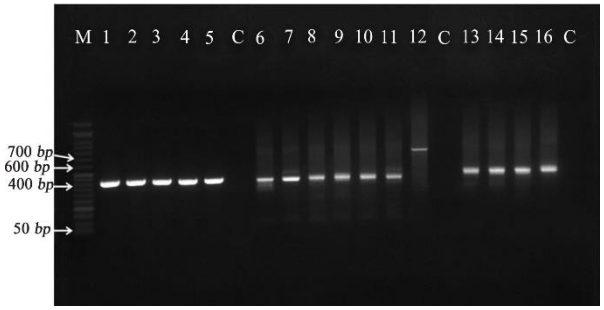

Figure 4. Agarose gel electrophoresis for mtDNA PCR products. Samples 1-5 for mtDNA confirmation using (F1-R1) mtDNA primers (Bands sizes of 414 *bp*). Samples 6-11: bands of 406 *bp* size for confirmation of 7599 *bp* deletion using (F4-R3) 7599 *bp* primers. Sample 12 indicates a band of 756 *bp* for the confirmation of 7599-*bp* deletion using (F2-R3)7599 *bp* primers. The last samples 13-16 indicate the bands of 555 *bp* for the confirmation of the 7345-*bp* deletion using (F5-R4) 7345-*bp* primers. M and C indicated DNA marker and negative controls, respectively

there was no significant difference in the presence of the 7345 *bp* mtDNA deletion in 41 infertile individuals (33.9%) compared to 30 fertile individuals (38.0%) of the control group (p=0.650) (OR=0.837, 95% CI=0.464-1.51) (Table 2).

Also, infertile and fertile individuals were sub-categorized into nine groups based on the sperm motility percentage as shown in tables 3 and 4. Using Spearman's correlation, the frequency of 7599 *bp* mtDNA deletion in the categorized groups showed a significant correlation between the sperm motility percentage and the presence of the 7599 *bp* mtDNA deletion (p=0.001, r=-0.887) (Table 3). However, the frequency of 7345 *bp* mtDNA deletion in the categorized groups showed no significant correlation between sperm motility percentage and the presence of the 7345 *bp* mtDNA deletion (p=0.98, r=0.008) (Table 4). Direct DNA sequencing was performed to verify the deletion sites in the mtDNA as shown in figures 5 and 6.

Collectively, the current cohort showed that the prevalence of 7599 *bp* mtDNA deletion was notably higher and significantly associated with asthenozoospermia-induced infertility.

Discussion

Asthenozoospermia is considered a major cause of male infertility worldwide that is attributed to

Table 4. Frequencies of 7345 *bp* mtDNA deletion in infertile and fertile group according to sperm motility

Motility	Number of cases	Presence of deletion	Percentage	p-value (spearman's correlation)
0%	20	9	45%	0.98 (r=0.008)
1-5%	20	7	35%	
6-10%	20	4	20%	
11-15%	20	4	20%	
16-20%	20	13	65%	
21-35%	21	4	19%	
50-55%	25	12	48%	
56-60%	27	12	44.4%	
61-75%	27	6	22.2%	
Total	200	71		

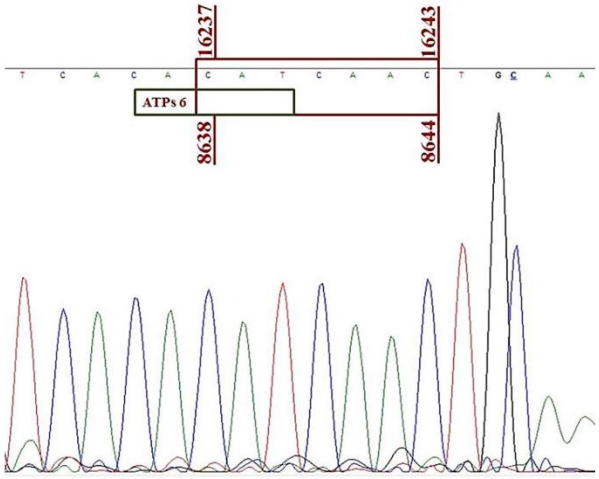

Figure 5. Sequencing result for sperm mtDNA PCR product for 7599 *bp* mtDNA deletion. The nucleotide sequence inside the red box indicated the seven nucleotide direct repeat at both sides of the deletion as indicated by the numbers above and below the box. ATPase 6 gene is included within the deletion

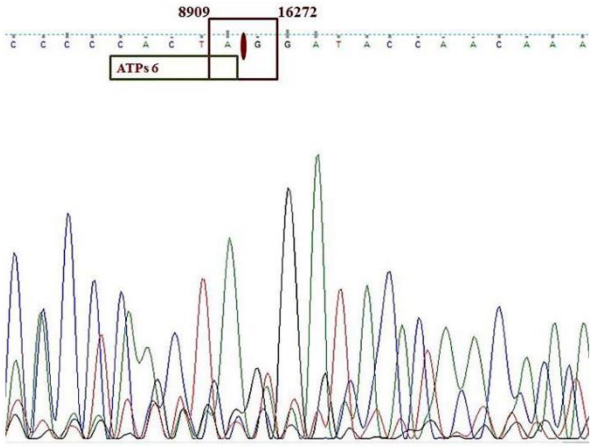

Figure 6. Sequencing result for sperm mtDNA PCR product for 7345 *bp* mtDNA deletion. The red arrow indicates the site of the deletion breakpoint. ATPase 6 is included within deletion

the insufficient provision of energy (ATP) to spermatozoa through the OXPHOS mechanism of the mitochondria located in the middle piece of the spermatozoon (7, 21). The mtDNA is responsible for encoding four of the five major complexes of OXPHOS complexes (22). Considering the presence of a single copy of the mtDNA in each mitochondrion (23), naturally, the mtDNA lacks efficient proofreading and repairing mechanisms, so the rate of mutation is much higher than the nuclear DNA (13). Simultaneously, the mtDNA is susceptible to be attacked by ROS which is generated from the normal mitochondrial respiration process, and as a consequence, the mtDNA may be damaged due to elevated oxidative stress which affects sperm functions mainly the sperm motility (24). Several mtDNA deletions have been reported to be highly correlated with male infertility in patients with asthenozoospermia, such as the 4977 *bp*, 7599 *bp*, and 7345 *bp* deletions. In a previous study, a significant association between the mtDNA 4977 *bp* deletion and the asthenozoospermia in Jordanian population was reported (25).

Additionally, a highly significant association was found between the presence of 7599 *bp* mtDNA deletion and the reduction in sperm motility in the same study. However, the 7345 *bp* mtDNA deletion did not show any significant correlation with men's infertility and asthenozoospermia. In addition, our DNA sequencing results confirmed the loss of essential genes that are responsible for the OXPHOS process. The current results are consistent with a recently published study in Iran (16). The researchers found a significant association between the incidence of 7599 *bp* mtDNA deletion and asthenozoospermia and also 7599 *bp* mtDNA deletion was found in the individuals with oligoasthenoteratozoospermia; the occurrence of this deletion is associated with the loss of the ATPase 6 gene in addition to the other OXPHOS genes of the mtDNA, which consequently affect the sperm motility. In addition, they did not find any significant association between the prevalence of 7345 *bp* deletion and reduction in sperm motility (16). These findings were supported by other researchers who found that the existence of large-scale mtDNA deletions such as 7599 *bp* and 7491 *bp* was markedly associated with reduced sperm motility because they are responsible for the loss of some mitochondrial genes affecting male fertility. They also proposed that screening mtDNA for such deletion may provide clear evidence behind the etiologies of male infertility (26). Moreover, the role of the 7599 and 7345 *bp* mtDNA deletions in male infertility was investigated by Kao et al. (9). They found a significant association between the incidence of 7599 *bp* deletion in the sperm mtDNA and the reduction in sperm motility which is supported by our findings (9).

Kao et al. reported a significant association between the incidence of 7345 *bp* deletion and asthenozoospermia (9) which is inconsistent with our results. Compared to our findings, the discrepancy in the results may be due to small sample size which consisted of 36 patients compared to 121 that were included in the current study.

However, they reported that these deletions are responsible for the loss of some genes in OXPHOS process. Such a loss will affect the activity of the mitochondria in energy production and therefore impairs sperm motility which as a consequence elevates the proportion of male infertility (9).

Moreover, the results of our study are inconsistent with the results of a research study conducted by John et al. (15). The existence of 7491 *bp* mtDNA deletion in spermatozoa was investigated in 33 semen samples. They proposed that using magnetic beads coated with a special antibody for the separation of sperm may help in the elimination of leukocytes which are considered as contaminants of sperm specimens. As a result, they suggested that there was no association between these mtDNA deletions and sperm count, morphology, or motility (15).

According to the current findings, the difference in the incidence of 7345 *bp* mtDNA deletion among the patients' group rather than control subjects does not necessarily mean that this deletion clinically contributes to sperm motility; in fact, partial correlation analysis revealed that 7599 *bp* mtDNA deletion is significantly correlated with motility, while 7345-*bp* mtDNA deletion is not significantly correlated with motility among study subjects. In support of that, the 7599 *bp* deletion is even more significantly correlated with motility when compared to 7345 *bp* deletion.

The contradiction in the results of various studies can be attributed to variations in the methodology, especially the sperm separation protocols. Moreover, the prevalence of the deletions in sperm mtDNA among control groups may be due to the effect of the environmental factors such as elevated scrotal temperature, the exposure to pesticides, radiation and cigarette smoking which lead to accumulation of the oxidative stress and therefore an increase in the chance of DNA breaks and damage. Also, some medications and antibiotics can affect male fertility. Furthermore, the sample size in the mentioned studies was small and the variation between populations may be a factor behind the contradictory results.

Therefore, the reported mtDNA deletions have to be studied on a larger scale population with consistent techniques of separation and processing to emphasize the role of these mutations in the development of male infertility. From the molecular point of view, the reported mutations are associated with the deletion of a large portion of mtDNA responsible for the expression of OXPHOS complexes. In light of that, it is crucial to clarify the presence of these large scale mutations in the control groups. Generally, the presence of these mutations in the control (Fertile) males can be attributed to the percentage of these mutations in the spermatozoa. That is supported by the correlation between the incidences of the mtDNA deletions and sperm motility.

Conclusion

A high prevalence of mtDNA deletion was reported in infertile Jordanian males in this study and a highly significant association was found between the presence of 7599 *bp* mtDNA deletion and asthenozoospermia. However, no significant correlation was reported between the presence of 7345 *bp* mtDNA deletion and asthenozoospermia. Our current findings support the previous report of the association between the mtDNA deletion and the occurrence of asthenozoospermia in Jordanian population. Further investigations are required to study other mtDNA mutations for their association with male infertility.

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Conflict of Interest

We declare there is no conflict of interest.

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