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Male infertility is significantly associated with multiple deletions in an 8.7-kb segment of sperm mtDNA in Pakistan

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Background/aim: This study aimed to find a link between sperm mitochondrial DNA mutations and male infertility in Pakistan.

Materials and methods: DNA from semen samples was extracted and amplified by PCR using 7.8-kb deletion-specific primers. The PCR products were separated on agarose gel, visualized under UV-illumination, and then photographed. The results were genotyped and the data were analyzed using SPSS.

Results: Deletion analysis of the 8.7-kb fragment by long PCR revealed multiple deletions. The frequency of deletion was much higher in infertile groups as compared to the control group. Further, on comparison between different subtypes of infertile groups, the deletions were highest in the oligoasthenoteratozoospermia (OAT) group. The statistical analysis of case and control groups showed a significant association of the 8.7-kb deletion with human male infertile groups (P = 0.031), and particularly a very significant association with the OAT subgroup (P = 0.019).

Conclusion: A significant association has been found between human male infertility and mtDNA deletions in an 8.7-kb segment of sperm mtDNA in a Pakistani population.

Key words: Human infertility, sperm, mtDNA 8.7 kb, multiple deletions

1. Introduction

Infertility is increasingly becoming a serious problem for the modern industrial human, affecting almost one out of every six couples. A similar scenario is observed in Pakistan. It is estimated that 20%–25% of infertility cases are due to male factors (1). About 13%–18% of couples suffer from it, and about one-half of all cases can be traced to either of the partners (2). Male infertility is more challenging to tackle compared to female infertility (3). Increasing evidence is available suggesting that male infertility has a genetic link (4,5). About 30% of men reporting to infertility clinics were found to have oligozoospermia, oligoasthenoteratozoospermia (OAT), or azoospermia of an unknown aetiology. In this scenario the missing link of male infertility was required to be identified. In infertile males, sperm motility is usually the foremost cause. The sperm motility may be linked to multiple mitochondrial DNA deletions. ATPs are the fuel to move sperm and help them reach the fallopian tubes, leading to fertilization (6). In the human body, every mitochondrion produces approximately 107 reactive oxygen species (ROS) per day. ROS have been reported to damage the mitochondrial and cellular proteins, lipids, and nucleic acids, as well as mtDNA, interrupting energy production (7,8). In asthenozoospermia many mtDNA mutations were found to be linked to ROS (9). This increases midpiece sperm defects and the acrosome reaction in affected sperm (10). A very high mtDNA mutation rate results in heteroplasmy, a mixture of mutant and wild-type DNA (11). A specific number (threshold) of mutant mtDNAs are required within a tissue for oxidative dysfunction. In this study we focused on a large 8.7-kb fragment of sperm mtDNA to amplify multiple mutations simultaneously. This fragment was supposed to be the most heavily muted out of the 16.6-kb genome. This large fragment of mtDNA consists of genes complex III (cytochrome b), complex IV (the cytochrome c oxidase gene, COXIII), and complex V, the ATPase synthase genes ATPase 6 and 8. A high incidence

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of deleted mtDNA was expected in the asthenozoospermic and oligoasthenozoospermic groups and an especially higher incidence of mutations was presumed in the OAT group.

We hypothesized that higher sperm mtDNA mutations in the infertile group are linked to infertility. More defective sperm (OAT type) was presumed to have a higher incidence of mutations as compared to the control group.

2. Materials and methods

2.1. Participants and study design

Carefully selected human semen samples (n = 203; 20–58 years of age, mean age 34.24 years) were collected, excluding leukospermia. Samples were divided into one control group and four infertile groups in accordance to the 2010 WHO Manual of Semen Analysis (12). Of the total 203 samples, 42 were in the control group and the infertile groups contained 161 in total. The research project was approved by the Research Ethics Committee of Quaid-e-Azam University Islamabad.

2.2. Laboratory examination

Semen DNA extraction was performed by modified phenol method (13,14) and quantified by a spectrophotometric method. Primers were designed using a reference human mitochondrial DNA sequence downloaded from the Entrez database (Accession Number NC_001807) and by applying the freely available Primer3 software program. MT1A as forward primer with fragment position 8, 224–8, 247: 5′ TCT AGA GCC CAC TGT AAA G 3′ and MT3 as reverse primer with its genomic position from 13, 580–13, 551: 5′ AGT GCA TAC CGC CAA AAG A 3′ were used for PCR amplification in this study. Each PCR reaction was performed in a total reaction volume of 10 µL. Each reaction was composed of 5.52 µL of deionized water, 1.0 µL of 10X PCR buffer with $MgCl_2$, 0.8 µL of dNTP, 0.08 µL of long PCR enzyme, 0.3 µL of each primer MT1A (20 µM) and MT3 (20 µM), and 2.0 µL of mtDNA sample (15). PCR amplification was performed with 1 cycle of initial denaturation at 94 °C for 2 min, followed by 34 cycles of denaturation at 94 °C for 10 s and annealing at 52 °C for 30 s. The last cycle and final extension was performed at 68 °C for 10 min. The PCR products were separated on 2 % agarose gel (Promega) prepared in 1X TBE buffer (Tris-borate-ethylenediaminetetraacetic acid buffer). The gels were stained with ethidium bromide (0.5 ng/mL concentration), visualized under UV-illumination, and then photographed. Genotyping was carried out and the data obtained were analyzed statistically.

2.3. Statistical analysis

Statistics were analyzed with IBM SPSS 20. The chi-square test was applied. $P < 0.05$ was considered as significant.

3. Results

Of the total 203 samples, 42 were in the control group and the infertile groups contained 161 in total. Proportions of MT1A and MT3 products were compared (control versus infertile groups). Figure 1 shows the number of bands, where the presence of the 8.7-kb band indicates wild-type DNA, the absence of the 8.7-kb band indicates deleted-type DNA, and the presence of the 8.7-kb band along with other bands indicates hybrid-type DNA. The genotypes of the control and the four infertile groups are given in Table 1 and Figures 2 and 3. A lower incidence of the wild-type band was found in the infertile groups as compared to the

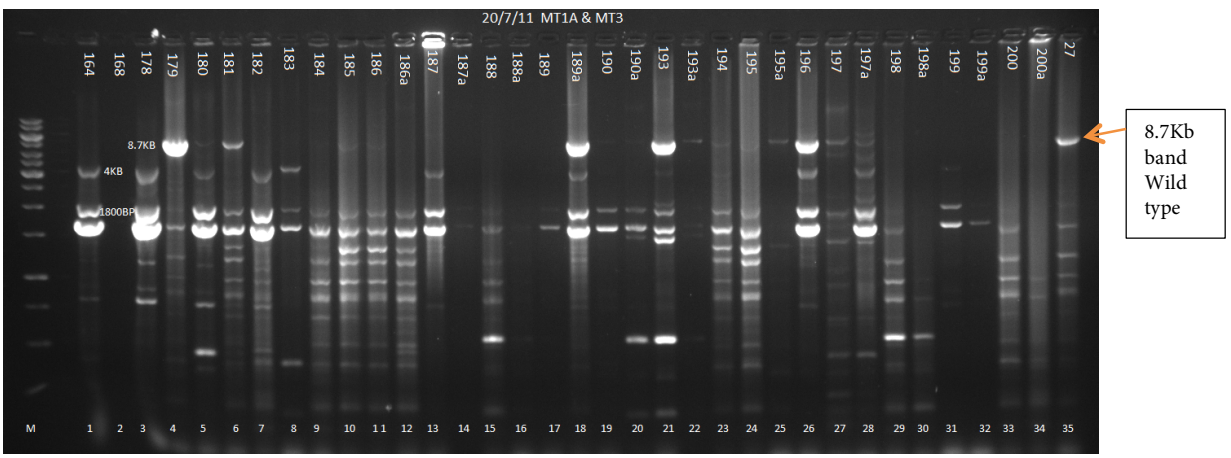

Figure 1. MT1A and MT3 electrophoretogram (mostly OAT-type). This gel is showing the total number of the samples (n = 35), where the control group sample (n = 1) shows up in lane 10, which is the hybrid type. The oligozoospermic sample (n = 1) is in lane 15, which is the deleted type, and the necrozoospermic sample (n = 1) is in lane 1, which is the muted type of DNA. All other samples are from the OAT group (n = 33). Lanes 22 and 25 are wild type. Lanes 1, 3, 7, 8, 9, 12, 13, 14, 16, 17, 19, 20, 22, 23, 24, 29, 30, 31, 32, 33, and 34 are the deleted type; lanes 4, 5, 6, 10, 11, 18, 21, 26, 27, 28, and 35 are the hybrid type.

Table 1. Proportion of MT1A and MT3 products in control versus infertile groups.

Status	Genotype, n (%)			Total	Chi-square df =10	P
	w	d	wd			
Control	5 (11.9)	23 (54.76)	14 (33.33)	42	29.85	0.001
Oligozoospermic	0 (0)	11 (50)	11 (50)	22		
Asthenozoospermic	0 (0)	19 (48.72)	20 (66.66)	39		
OAT	1 (1.04)	64 (66.67)	31 (32.29)	96		
Necrozoospermic	0 (0)	2 (50.0)	2 (50.0)	4		
Total	6 (2.95)	119 (58.62)	78 (38.42)	203		

W = Wild 8.7 kb, d = deleted absence of 8.7 kb, wd = hybrid (multiple bands simultaneously). OAT: Oligoasthenoteratozoospermia.

control group. The infertile groups had a higher incidence of deletions. The highest incidence of deletions was observed in the OAT group. This analysis demonstrated a highly significant association (P = 0.001) of multiple deletions in the infertile groups. Proportions of the 8.7-kb fragment in the control and infertile groups are given in Table 2 and Figure 4. Infertile groups had very high incidences of the hybrid type (n = 64) as compared to the wild type (n = 1) and the highest incidence of the deleted type (n = 96). This indicated a significant association (P = 0.031) of deletions with infertility. Proportions of the 8.7-kb fragment for the control versus the OAT group are given in Table 3 and Figure 5. The OAT group had the highest incidence of deletions; the wild type (n = 1) was 1.04%, deleted (n = 64) was 66.67%, and hybrid (n = 31) was 32.29%. This indicated a strong association (P = 0.019) of deletions in the OAT group. Overall analysis of data from all groups revealed a highly significant association (P = 0.001).

4. Discussion

Amplification and evaluation of the 8.7-kb segment of sperm mtDNA revealed multiple deletions. Only the control group revealed a higher incidence of wild-type DNA. The infertile groups revealed higher incidences of muted DNA and of hybrid-type DNA. The OAT group exhibited the highest incidence of muted and hybrid types of DNA (Table 1). The analysis of results indicated an association of mutations with the infertile groups. The overall chi-square test determined a significant difference (P = 0.001). The results support our hypothesis that 8.7-kb deletions are directly proportional to infertility. When we analyzed all infertile groups in comparison to the control group, the results had the same patterns (Table 2). Higher incidences of muted and hybrid types of DNA were found in the infertile groups as compared to the control group. The chi-square test (P = 0.031) indicated a significant relation of mutations with infertility. Statistical analysis of the OAT group was also compared with the control group (P = 0.019) (Table 3). The overall trend of our results supports our hypothesis that sperm mtDNA mutations are related to sperm morphological abnormalities leading to human male infertility.

Previous studies had a vacuum to fill with regards to the size and relevancy of their samples, hence postulating

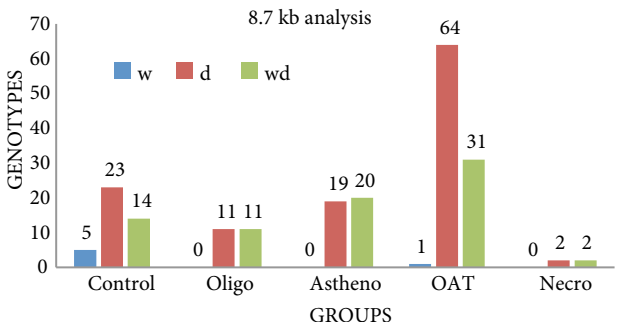

Figure 2. Proportion of MT1A and MT3 products in control versus infertile groups.

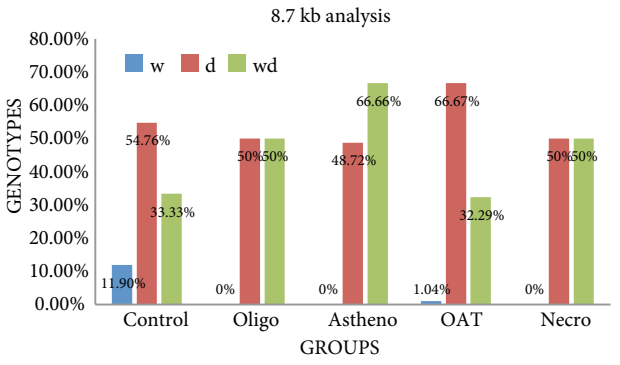

Figure 3. Percentage of 8.7-kb segment in control versus infertile groups.

Table 2. Analysis of 8.7-kb fragment in control versus infertile groups.

Status	Genotype, n (%)			Total	Chi-square df =2	P
	w	d	wd			
Control	5 (11.9)	23 (54.76)	14 (33.33)	42	6.970	0.031
Infertile groups	1 (0.62)	96 (59.01)	64 (39.75)	161		
Total	6 (2.95)	119 (58.62)	78 (38.42)	203		

W = Wild 8.7 kb, d = deleted absence of 8.7 kb, wd = hybrid (multiple bands simultaneously).

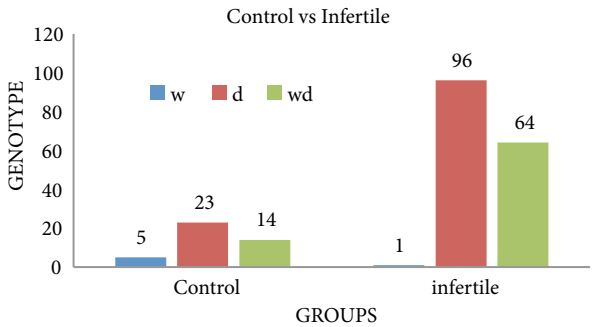

Figure 4. Proportion of 8.7-kb fragment in control and infertile groups.

results that were not this clearly defined. Men with OAT harbor higher numbers of multiple mitochondrial DNA deletions in their spermatozoa, but individual deletions are not indicative of overall aetiology; rather, multiple deletions are (16). Mitochondrial DNA integrity and its copy number are associated with infertility (17). The relevance of the predictive nature of multiple ΔmtDNA was important considering the composition of patients seeking medical help for infertility. One study indicated that 15% of patients attending infertility clinics were suffering from OAT syndrome (18), and this was the fraction of infertile patients who were supposed to have the highest number

Table 3. Analysis of 8.7-kb fragment in control versus OAT group.

Status	Genotype, n (%)			Total	Chi-square df =2	P
	w	d	wd			
Control	5 (11.9)	23 (54.76)	14 (33.33)	42	7.899	0.019
OAT	1 (1.04)	64 (66.67)	31 (32.29)	96		
Total	5 (3.79)	88 (66.67)	45 (34.09)	132		

w = Wild 8.7 kb, d = deleted absence of 8.7 kb, wd = hybrid (multiple bands simultaneously).

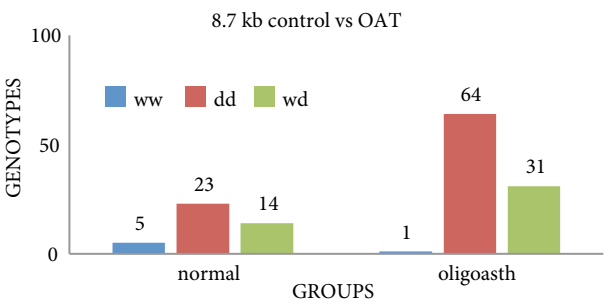

Figure 5. Proportion of 8.7-kb fragment in control versus OAT group.

of mutations. It was observed that random deletions of different sizes were indicative of poor sperm quality (19). ROS were proved as the foremost cause of depletion of sperm quality and survivability (20). A definite association of ROS and mtDNA mutations during spermatogenesis was established (21). If deletions are already present in the germinal layer of the testes, spermatogenesis would produce very low numbers of sperm as well as the highest ratio of mutations, quite similar to that found in OAT. Increasing numbers of mutations can be found in the case of a spermatogonial defective background, favoring the amplification of deleted molecules (22). Control group subjects also revealed multiple deletions and hybrid DNA. A possible explanation for these mutations in such cases

is the heteroplasmic nature of mtDNA, as well as stem cells with excessive deletions acting as a catalyst to such a scenario. Numbers of mutations in the control group were far lower than in the OAT group, indicating a proportionate relation with infertility. These deleted molecules might have been preferentially selected, but sufficient intact molecules would still be generated to account for the patients with normal characteristics, i.e. they were not significant enough to hamper motility or cause such abnormalities in spermatozoa. The electron transport chain mechanism and its ATP production for sperm motility tend to be species-specific (23). Mitochondrial DNA integrity was found depleted in oligozoospermia (15). To this extent, cell model systems were required to determine how these important relationships function (24). This was a further indication that in these patients who may be candidates for intracytoplasmic sperm injection, the spermatozoa may be harboring genetic defects with bad prognosis for forthcoming conceptions. It was already observed that the effect was not on the number of mitochondria themselves but rather on the number of mtDNA molecules per mitochondrion, which were reduced mostly by different pathophysiological conditions (24,25). Exogenous sources of ROS or free radicals may aggravate oxidative damage, resulting in mutations of mtDNA in human tissues (26,27).

Our study and statistical analysis conclude with an association of infertility with 8.7-kb segment mtDNA sperm deletions, particularly more evident in the OAT group (oligoasthenoteratozoospermic). Simple semen analysis detecting sperm abnormalities like OAT, teratozoospermia, and asthenozoospermia can be a raw indicator of sperm mitochondrial DNA abnormalities and can be a basic first-assessment tool of sperm qualities.

References

1. De Kretser DM, Baker HWG. Infertility in men: recent advances and continuing controversies. J Clin Endocr Metab 1999; 84: 3443-3450.
2. Seshagiri PB. Molecular insights into the causes of male infertility. J Biosci 2001; 26: 429-435.
3. Cummins JM, Jequier AM, Kan R. Molecular biology of human male infertility: links with ageing, mitochondrial genetics, and oxidative stress? Mol Rep Dev 1994; 37: 345-362.
4. Holyoake AJ, McHugh P, Wu M, Carroll OS, Benny P, Sin IL, Sin FY. High incidence of single nucleotide substitutions in the mitochondrial genome is associated with poor semen parameters in men. Int J Androl 2001; 24: 175-182.
5. Spiropoulos J, Turnbull DM, Chinnery PF. Can mitochondrial DNA mutations cause sperm dysfunction? Mol Hum Reprod 2002; 8: 719-721.
6. Lamirande E, Gangon C. Reactive oxygen species and human spermatozoa II. Depletion of adenosine triphosphate plays an important role in the inhibition of sperm motility. J Androl 1992; 13: 379-377.
7. Attardi G, Schatz G. Biogenesis of mitochondria. Annu Rev Cell Biol 1988; 4: 289-333.
8. Wallace DC, Brown MD Brown, Lott MT. Mitochondrial DNA variation in human evolution and disease. Gene 1999; 238: 211-230.
9. Wallace DC. Diseases of the mitochondrial DNA. Annu Rev Biochem 2005; 61: 1175-1212.
10. John JC, Jokhi RP, Barratt CL. Men with oligoasthenoteratozoospermia harbor higher numbers of multiple mitochondrial DNA deletions in their spermatozoa, but individual deletions are not indicative of overall aetiology. Mol Hum Reprod 2001; 7: 103-111.
11. DiMauro S, Schon EA. Mitochondrial respiratory chain disease. N Engl J Med 2003; 348: 2656-2668.
12. World Health Organization. Manual for the Examination and Processing of Human Semen. 5th ed. Geneva, Switzerland: WHO; 2010.
13. Kao SH, Chao HT, Wei YH. Mitochondrial deoxyribonucleic acid 4977 bp deletion is associated with diminished fertility and motility of human sperm. Biol Reprod 1995; 52: 729-736.
14. Sambrook J, Russell D. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press; 2001.
15. Lestienne P, Reynier P, Chretien MF, Penisson-Besnier I, Malthiery Y, Rohmer V. Oligoasthenospermia associated with multiple mitochondrial DNA rearrangements. Mol Hum Reprod 1997; 3: 811-814.
16. St John JC, St John B. Sperm mitochondrial DNA. In: Zini A, Agarwal A, editors. Sperm Chromatin. Berlin, Germany: Springer; 2011. pp. 81-94.
17. Song JG, Lewis V. Mitochondrial DNA integrity and copy number in sperm from infertile men. Fertil Steril 2008; 90: 2238-2244.
18. Irvine DS. Epidemiology and etiology of male infertility. Human Reprod 1998; 13: 33-44.
19. Reynier P, Chretien MF, Savagner F, Larcher G, Rohmer V, Barriere P, Malthiery Y. Long PCR analysis of human gamete mtDNA suggests defective mitochondrial maintenance in spermatozoa and supports the bottleneck theory for oocytes. Biochem Biophys Res Commun 1998; 252: 373-377.
20. de Lamirande E, Leciere P, Gagnon C. Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. Mol Hum Reprod 1997; 3: 175-194 .

21. Cummins JM, Jaquier AM, Martin R, Mehmet D, Goldblatt J. Semen levels of mitochondrial DNA deletions in men attending an infertility clinic do not correlate with phenotype. Int J Androl 1998; 21: 47-52.

22. St John JC, Sakkas D, Barrat CLR. A role for mitochondrial DNA and sperm survival. J Androl 2000; 21: 189-199.

23. Storey BT. Strategy of oxidative metabolism in bull spermatozoa. J Exp Zool 1980; 212: 61-67.

24. Larsson NG, Oldfors A, Holme E, Clayton DA. Low levels of mitochondrial transcription factor A in mitochondrial DNA depletion. Biochem Biophys Res Commun 1994; 200: 1374-1381.

25. Bentlage HACM, Attardi G. Relationship of genotype to phenotype in fibroblast-derived transmitochondrial cell lines carrying the 3243 mutation associated with the MELAS encephalomyopathy: shift towards mutant genotype and role of mtDNA copy number. Hum Mol Genet 1996; 5: 197-205.

26. Yang JH, Lee HC, Wei YH. Photoageing-associated mitochondrial DNA length mutations in human skin. Arch Dermatol Res 1995; 287: 641-648.

27. Fahn HJ, Wang LS, Kao SH, Chang SC, Huang MH, YH Wei. Smoking-associated mitochondrial DNA mutations and lipid peroxidation in human lung tissues. Am J Respir Cell Mol Biol 1998; 19: 901-909.