ORIGINAL ARTICLE

Sperm mitochondrial DNA 15bp deletion of cytochrome c oxidase subunit III is significantly associated with human male infertility in Pakistan

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Abstract

Objective: To find genetic association of sperm mitochondrial deoxyribonucleic acid cytochrome c oxidase III subunit 15bp deletion with male infertility in Pakistan.

Methods: The case-control study was conducted from July 2011 to December 2013, and comprised semen samples that were divided into two main groups; the control group had normozoospermic patients while the other group had infertile subjects. The Infertile group was sub-divided into four groups on the basis of semen analysis. Deoxyribonucleic acid was extracted using modified organic extraction method, amplified by polymerase chain reaction with cytochrome c oxidase III-specific primers. The fragments were separated by agarose gel electrophoresis; 135bp wild fragment and 120bp deleted one. Data was analysed using SPSS 22.

Results: Of the 194 samples, 44(22.6%) were controls, and 150(77.3%) were infertile. The infertile group sub-division was oligozoospermic 20(13.3%), asthenozoospermic 36(24%), oligoasthenoteratozoospermic 88(58.6%) and necrozoospermic 6(4%). Polymerase chain reaction amplification of the control group revealed wild 4(9.09%), deleted 13(29.55%) and hybrid 27(61.36%)The findings in the four infertile sub-groups were: deleted 6(30%) and hybrid 14(70%) in oligozoospermic, deleted 12(33.33%) and hybrid 24(66.66%) in asthenozoospermic, wild 2(2.27%), deleted 41(46.59%) and hybrid 45(51.14%) in oligoasthenoteratozoospermic, and wild 1(16,66%) and hybrid 5(83.33%) in the necrozoospermic group. There was a significant association of cytochrome c oxidase III 15bp deletion with human male infertility (p=0.033).

Conclusion: There was a higher frequency of mutations in infertile groups compared to the control group.

Keywords: Male infertility, mtDNA, deletion, PCR, COXIII. (JPMA 66: 3; 2016)

Introduction

Infertility is an increasingly reported problem and 10-15% of couples in total are affected and 50% cases can be traced to either of the partners.[1] It is estimated that 20-25% of infertility cases are due to male factors.[2] Male infertility is more difficult to describe compared to female infertility.[3] Evidence suggests that male infertility has a genetic cause.[4,5] Unknown aetiology is found in almost half of infertile reported cases and can be congenital or acquired. About 30% of men reporting to infertility clinic are found to have oligozoospermia, oligoasthenoteratozoospermia (OAT) and orazoospermia of unknown aetiology. Male infertility is primarily related with sperm count and motility disorders, which is linked to mitochondrial deoxyribonucleic acid (mtDNA) anomalies. Adenosine triphosphates (ATPs) are the major available fuel for sperms to move and reach fallopian tubes for successful fertilization.[6] Oxidative phosphorylation that produces the reactive oxygen species (ROS) can damage mitochondrial and cellular proteins, lipids, the nucleic acids, as well as mtDNA, interrupting energy production.[7] High incidence of the nucleotide substitutions in mitochondrial genome is found associated with poor semen quality.[8] Many mutations in mtDNA, which are associated with asthenozoospermia are due to ROS.[9] Oxidative stress (OS) plays a massive role in the aetiology of total defective sperm count.[10] This in turn increases mid-piece sperm defects and the acrosome reaction.[11] Normally, all mtDNA in a cell are identical (homoplasmic), but due to very high mutations rate in mtDNA, it becomes a mixture of mutant and wild type DNA termed heteroplasmy. A mixed variety of wild and mildly deleterious mutations of mtDNA are found in human populations, causing a heterogeneous group of disorders with a broad spectrum of clinical phenotypes and variable severity.

In mitochondria, oxidative phosphorylation occurs through an electron transport chain. Cytochrome c oxidase (COX) transfers electrons from reduced cytochrome c to molecular oxygen. The human cytochrome consists of 13 subunits; among them the three largest are encoded by mtDNA are COXI, COXII, and COXIII.[12] The COXIII gene mutation results in complete

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and partial respiratory chain defects and complete loss of activity has been observed in cells with homoplasmic COXIII mutation. The homoplasmic15bp deletion results in abolishing the activity of COXI, COXII and COXIII subunits in holoenzyme as all the subunits are related and dependent on the COXIII subunit.[13]

We hypothesized that sperm mtDNA is vulnerable to damage and mutations may be associated to infertility. The study was planned to focus on15bp deletion in COXIII subunit of mtDNA in Pakistani infertile patients. This selection of 15bp deletion of mtDNA was based on the observation of its important role in sperm motility and fertility.

Materials and Methods

The case-control study was conducted from July 2011 to December 2013, and comprised semen samples that were divided into two main groups; the control group had normozoospermic subjects, while the other group had infertile patients. The Infertile group was sub-divided into four groups on the basis of semen analysis.

After approval by the Research Ethics Committee of the Quaid-i-Azam University, Islamabad, human semen samples were collected in line with the World Health Organisation (WHO) guidelines.[14] All the controls had at least one child, whereas infertile individuals were cases of idiopathic infertility.

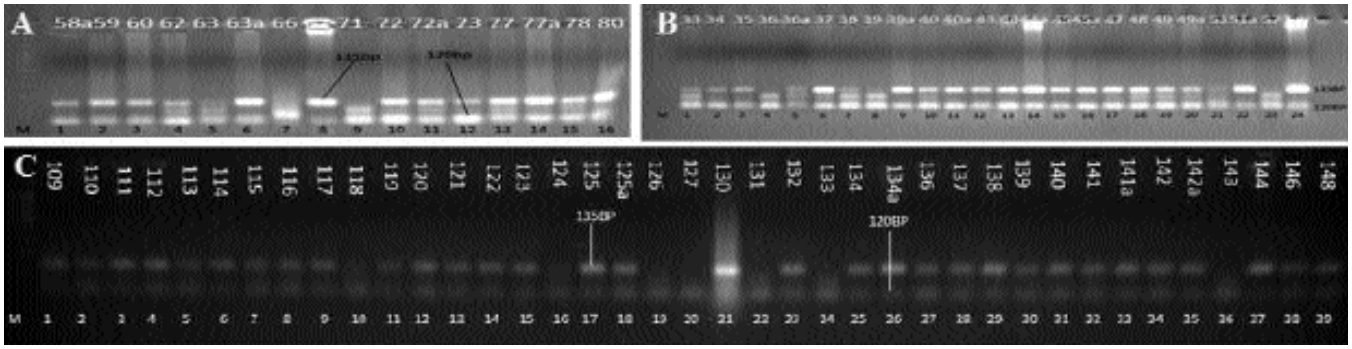
The DNA from semen samples was extracted by standard organic protocol of DNA extraction, except that the samples were supplemented with 1.5µl of 20mg/ml Proteinase K (GeneDireX, Germany) and 12µl of 1 mol/l dithiothreitol (Sigma, UK) at the step of cell lysis and incubation at 55°C.[15] The DNA quantification was carried out spectrophotometrically using 260nm/280nm wavelengths.

Polumerase chain reaction (PCR) amplification was carried out using COXIII, 15bp using 9390 to 9413: 5' ACA CGA GAA AGC ACA TAC CAA GGC 3' and 9525 to 9502: 5' CTA GGC TGG AGT GGT AAA AGC CTC 3' as forward and reverse primers respectively. Each 10µl PCR reaction consisted of 1µl of 10X PCR buffer with Magnesium chloride (MgCl2), 0.8µl 2mM deoxynucleotide triphosphates (dNTPs), 0.2µl of 5U Taq DNA polymerase enzyme (Fermentas, Lithuania), 0.3µl of 20µM of each forward and reverse primers, and 2µl of 40ng/ µlDNA. Thermal cyclic conditions were: initial denaturation of 92°C for 3 minutes, followed by 25 cycles of denaturation at 92°C for 20 seconds, annealing at 62°C for 35 second and extension at 72°C for 3 minute.

The amplified PCR products were separated on agarose gels along with 100bp DNA ladder (Fermentas) as the standard size. The gel was run for 120 minutes using the Maxicell EC360-M electrophoretic gel system (EC Apparatus Corporation, St. Petersburg, Florida, USA) at 200 constant volts by using BioRad Power Pack 3000 (BioRad, USA) in 1X TBE buffer. The gels were stained with ethidium bromide and photographed under ultraviolet (UV) transillumination. Data was compiled and analyzed using SPSS 22. Chi Square test was applied and P value less than 0.05 was considered significant.

Results

Of the 194 samples, 44(22.6%) were controls, and



COX-III: Cytochrome c oxidase III. mtDNA: Mitochondrial deoxyribonucleic acid. OAT: Oligoasthenoteratozoospermic.

Figure-1: Electrophoretograms: A) Figure demonstrates amplification of normozoospermic sample by COXIII specific primers. 135bp band is wild type and 120bp band is indicative of mtDNA with 15bp deletion. Sample lane 7 and 9 are homogenous deleted type (120bp) and lane no: 1,2,3,5,6,8,10,11,12,13,14,15 are hybrid. Lane 16 is control. A 250bp laddar was used. B) Figure shows 24, while normozoospermic lane 23 is muted; 01 OAT group with lane 3 being the hybrid type; 21 asthenozoospermic with lanes 4, 8 and 21 as well as lanes 1,2,5,6,7,9,10,11,12,13,14,15,16,17,18,19,20 and 22 being muted, and lane 24 being control. C) Figure shows 5 asthenozoospermic with lane 19 being muted and lanes 1,2,3,4 being hybrid; 16 pligozoospermic with lane 16 and 24 being muted and lanes 7,8,11,16,17,24,25,26 being hybrid; 22 OAT (oligoasthenoteratozoospermic) with lanes 10,20,22,36 being muted and lanes 5,6,9,12,13,14,15,18,23,27,28,29,30,31,32,33,34,35,37,38,39 being hybrid; and necrozoospermicwith lane 21 being the wild type.

Table-1: Clinical Data.

(a)	Control	Oligozoospermia	Asthenozoospermia	OAT	Necrozoospermia
Number	44	20	36	88	6
Age (years)	31.84±7.09	33.90±8.94	33.76±5.92	34.48±6.51	36.33±11.76
Count (millions/ml)	68.18±10.86	16.66±3.88	46.22±11.76	14.89±4.10	35.00±12.25
Vitality (%)	69.11±7.64	66.66±5.42	63.92±4.82	12.58±3.90	12.50±4.79
Progressive (%)	44.20±6.39	40.71±5.18	18.92±6.99	19.83±6.76	7.5±2.5

OAT: Oligoasthenoteratozoospermic.

Table-2: Comparison of weight, height and ethnic groups.

(b)		Group					Total
		Control	Oligo	Astheno	OAT	Necro	
Weight	Average	26	10	16	46	3	101
	Fatty	1	1	7	17	1	27
	Lean	17	9	13	25	2	66
Height	Average	35	15	27	64	3	144
	Small	6	1	3	7	1	18
	Tall	3	4	6	17	2	32
Ethnic	Other	6	0	3	6	0	15
	Pathan	6	5	7	19	1	38
	Punjabi	31	14	26	63	5	139
	Sindhi	1	1	0	0	0	2
Total in each category		44	20	36	88	6	194

OAT: Oligoasthenoteratozoospermic.

150(77.3%) were infertile. The overall mean age of the subjects was 34.05±0.619 years (range: 20-58 years) (Table-1). The infertile group sub-division was oligozoospermic 20(13.3%), asthenozoospermic 36(24%), OAT 88(58.6%) and necrozoospermic 6(4%). All the groups were compared for weight, height and ethnicity (Table-2).

Polymerase chain reaction amplification of the control group revealed wild 4(9.09%), deleted 13(29.55%) and hybrid 27(61.36%)The findings in the four infertile sub-groups were: deleted 6(30%) and hybrid 14(70%) in oligozoospermic, deleted 12(33.33%) and hybrid 24(66.66%) in asthenozoospermic, wild 2(2.27%), deleted 41(46.59%) and hybrid 45(51.14%) in OAT, and wild 1(16,66%) and hybrid 5(83.33%) in the necrozoospermic group. There was a significant association of COXIII 15bp deletion with human male infertility (p=0.033) (Table-3).

Discussion

Sperm mtDNA mutations and deletions abolish whole path of ATP production, depleting the movement, the vehicle of fertility. Individually, each multiple deletions of mtDNA are poor predictors of the overall incidence of deletion.[16,17] The multiple mtDNA mutations observed in different fragments have direct association with poor semen quality. Highest incidence of deletions was noted in OAT group. OAT syndrome renders sperm to lose motility and fertility. Semen parameters can be a raw predictive element of the nature of sperm quality. OAT syndrome does indicate the worst possible motility anomalies related to mutations leading to infertility. The relevance of the predictive nature of multiple mtDNA deletions is important considering the composition of patients seeking medical help for infertility. A study indicated that 15% patients attending infertility clinics are suffering from OAT syndrome.[18] This is the fraction of the infertile supposed to be present with maximum incidence of mutations in different segments of their mitochondrial genome. Idiopathic OAT affects approximately 30% of all

Table-3: Sperm deletion analysis.

Group	Genotype n (%)			Total	Chi-square	p
	ww	dd	wd		df=8	
Control	4(9.09)	13(29.55)	27(61.36)	44	18.46	0.033
Oligozoospermic	0(0)	6(30)	14(70)	20		
Asthenozoospermic	0(0)	12(33.33)	24(66.66)	36		
OAT (oligoasthenoteratozoospermic)	2(2.27)	41(46.59)	45(51.14)	88		
Necrozoospermic	1(16.66)	0(0)	5(83.33)	6		
Total	7(3.61)	72(37.11)	115(59.28)	194		

ww: Wild 135bp
dd: Deleted 120bp
wd: Hybrid.

infertile men.[19] It is observed that random mtDNA deletions are indicative of defective sperm quality.[20] COXIII subunit of COX of mtDNA has been focused by only a few previous studies.[21] The previously known sperm mtDNA male infertility researches produced vacuum for more research work. This study has the representation of affected individuals related to all the major ethnicities of Pakistan.

In this study 50bp upstream to 9480del15 amplification showed matching trend of deletions in different infertile groups. Comparatively more wild type mtDNA were found in control group and more incidence of deletions in infertile groups whereas highest incidences of deletions was in OAT group. The results were significant a (p=0.033).

A link is now associated between ROS and mtDNA mutations.[2] These ROSs are not the only mutant event initiator of the vicious circle leading to the complete halt of sperm motility and life.[22] Intrinsic anomalies during the process of spermatogenesis may be parting role in mutations.[23] If deletions are already present in germinal layer of testis, spermatogenesis would produce very low number of normal sperm and highest ratio of mutations similar to OAT syndrome.

The increasing number of mutations harbour in case of spermatogonial defective background, favouring the amplification of deleted molecules.[9] Our study proved that the incidence of mutation was more in OAT syndrome compared to the control group. To devise a relationship of deleted mtDNA and its final effect on spermatozoa and mitochondrial basic function, further considerable studies are.[9] Studies for mtDNA diseases were found to have definite loss of mitochondrial functions in those tissues and sperm mitochondria.[24] It was observed in oligozoospermia that mtDNA integrity was compromised.[25] Cell models systems are required to determine how these important relationships function.[26] This is a further indication that in these patients, who are maybe candidates for intracytoplasmic sperm injection (ICSI), the spermatozoa may harbour genetic defects with bad prognosis in forthcoming conceptions. It is already established that oxidative damage to lipids and DNA of sperm is associated with decreased motility and reduced fertility of spermatozoa.[27,28] Damage to mtDNA by oxidation is shown to be much higher than the damage to somatic DNA in humans.[29,30] In this study, we detected that common deletions were associated with poor semen quality. The multiple deletions simultaneously may cause complete removal or truncation of some basic genes and transfer ribonucleic acid (tRNA) genes of mtDNA. This in term resulted in muted DNA, defective subunits and impaired enzymes, and, finally, loss of respiration. Muted mtDNA results in reduced production of ATP. Vicious circle of increased formation of ROS as well as more free radicals leads to increasing damage to mtDNA, leading to immobility and infertility. This study is indicative of the link between depletions of sperm mtDNA and reduced sperm motility and fertility.

Studies indicate that mtDNA depletion is not because of the defective mitochondrial biogenesis. This is observed that the effect is not on number of mitochondria itself, rather on the number of mtDNA molecules per mitochondrion are reduced mostly by different pathophysiological conditions.[31]

It has been already established that in mtDNA mutations, deletions are involved at high frequency in sperm with motility disorders.[25,28] We are further focusing on multiple sperm mtDNA deletions to get the full spectrum of mtDNA deletions associated with male infertility in Pakistani population. It will help us to understand the role of genes located on mtDNA in infertility and disease mechanism.

Conclusion

The mtDNA deletions had significant impact on sperm quality, leading to infertility by affecting various sperm motility parameters, which are important determinants of male fertility. The observation support the hypothesis that mtDNA deletions have significant association with male infertility in Pakistan.

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