

Large Scale 7436-bp Deletions in Human Sperm Mitochondrial DNA with Spermatozoa Dysfunction and Male Infertility

PRAFULLA S. AMBULKAR¹, JWALANT E. WAGHMARE², AJAY R. CHAUDHARI³, VANDANA R. WANKHEDE⁴,
AADITYA M. TARNEKAR⁵, MORESHWAR R. SHENDE⁶, ASOKE K. PAL⁷

ABSTRACT

Introduction: Mitochondria and mitochondrial DNA are essential to sperm motility and fertility. It controls growth, development and differentiation through oxidation energy supply. Mitochondrial (mtDNA) deletions or mutation are frequently attributed to defects of sperm motility and finally these deletions lead to sperm dysfunction and causes infertility in male.

Aim: To investigate the correlation between large scale 7436-bp deletions in sperm mtDNA and non-motility of sperm in asthenozoospermia and Oligoasthenoteratozoospermia (OAT) infertile men.

Materials and Methods: The present prospective study was carried out in Human Genetic Division, Department of Anatomy, Mahatma Gandhi Institute of Medical Sciences, Sevagram from June 2014 to July 2016. We have studied 110 asthenozoospermia and OAT infertile men whose semen profile indicated abnormal motility and 50 normal fertile controls. Of 110 infertile men, 70 had asthenozoospermia and 40 had OAT. Fractionations of spermatozoa were done in each semen sample on the basis

of their motility by percoll gradients discontinuous technique. Long-range PCR was used for detection of 7436-bp deletions in sperm mtDNA and was confirmed by primer shift technique.

Results: Overall eight subjects (8/110; 7.2%) of which six (6/70; 8.57%) asthenozoospermia and two (2/40; 5%) OAT had shown deletions of 7436-bp. In 40% percoll fraction had more non-motile spermatozoa than 80% percoll fraction. The non-motile spermatozoa in 40% percoll fractions showed more mtDNA deletions (7.2%) than the motile spermatozoa in 80% percoll fraction (2.7%). The sequencing of flanking regions of deleted mtDNA confirmed 7436-bp deletions. Interestingly, no deletions were found in control subjects.

Conclusion: Though, the frequency of 7436-bp deletions in sperm mtDNA was low in infertile cases but meaningful indications were there when results were compared with controls. It is indicated that large scale deletions 7436-bp of mtDNA is associated with abnormal sperm motility. The 7436-bp deletions of mtDNA in spermatozoa may be one of the important causes of dysfunction and non-motile sperm.

Keywords: Asthenozoospermia, Mitochondrial DNA deletions, Oligoasthenoteratozoospermia

INTRODUCTION

Sperm motility is essential factor of fertile men. During fertilization, sperm cell require large amount of energy for their movement of flagella and active functioning. Nearly, 100 mitochondria are present in the midpiece of every mature human spermatozoon to provide energy quickly and effectively for sperm motility [1]. The oxidative phosphorylation of mitochondria generates energy in the form of ATP for flagellar movement of spermatozoa. In mitochondria, Reactive Oxygen Species (ROS) is generated during oxidative phosphorylation and increase the risk of Mitochondrial (mtDNA) damage [2]. The oxidative phosphorylation comprises a series of protein complexes that are encoded by both nuclear genes and mitochondrial genes [3]. Mitochondria contain their own genomic DNA and express independently in matrix of mitochondria. It contains 16569 base pairs that categorised in 13 genes of respiratory chain complex subunits, along with the 22 tRNAs and 2 rRNAs (12S and 16S) involved in protein synthesis [4]. The mtDNA replicates rapidly by D-loop mechanism without proof-reading and DNA repair mechanisms. So, it enhance mutation rate 10-100 times higher than that of nuclear DNA [5]. Furthermore, sperm cells are susceptible to damage from oxidants because they lack endogenous antioxidants activity and mtDNA is attached to the mitochondrial inner membrane where ROS are continuously generated as byproducts of electron

transport chain [6]. Higher frequencies of mtDNA deletions/ mutation have been identified in mitochondrial genome of affected tissues of patients and mitochondrial diseases [5].

The large scale "common" deletions of 4977 bp is the most identified cause in affected tissue of about 40% of patients with mitochondrial myopathy and related disorder [5]. The increased number of various large scale deletions such as "common" 4977-bp, 7436-bp and 7345-bp are well recognized to be associated with aging in various human tissues [7]. Kao et al., first correlated the association of the 4977-bp deletion of mtDNA with low motility of the human spermatozoa [8]. In our previous study, we also found correlation of the 4977-bp deletions of mtDNA with decrease motility of the human spermatozoa and infertility [9]. Several studies have also shown that multiple mtDNA deletions are strongly associated with defective sperm function and diminish fertility in men and these mutations cause infertility by affecting sperm motility [10,11]. On the other hand, low level of large scale deletions has been identified in human sperm mtDNA except 4977-bp large-scale deletions and not found a direct correlation between large-scale mtDNA deletions and male infertility [12]. Therefore, we investigated the correlation between large scale 7436-bp deletions in sperm mtDNA and non-motility of sperm in asthenozoospermia and Oligoasthenoteratozoospermia (OAT) infertile men.

MATERIALS AND METHODS

Collection and characterisation of Human Spermatozoa

Semen samples were collected from proven 70 asthenozoospermic and 40 OAT infertile men who attended our institutional reproductive biology laboratory of Department of Physiology, MGIMS, Sevagram. Recruitment of asthenozoospermia, OAT infertile men and 50 normozoospermic fertile men taken as control were done accordance with WHO 2010 guideline [13]. Semen samples were collected by masturbation in sterile container after 3-4 days of abstinence and kept at 37°C for 30 min, allowed to liquefy. Semen analysis was done according to WHO 2010 guideline including motility, characteristic, concentration and morphology [13]. Asthenozoospermia sample was considered if spermatozoa with <20% normal motility and OAT was consider with <20million/ml, <20% motility and <15% normal morphology of spermatozoa. Normozoospermic semen sample was taken with count >20 million/ml, >20% motility and >15% normal morphology of spermatozoa. All subjects were aged <40 years and included after physical examination and history taking. Infertile men with varicocele, cryptorchidism, testicular-pathy, vasectomy, long term medical history with medication, cigarette smoking and alcohol intake were excluded from our study. Informed consent form was obtained from each subject and study was approved by Mahatma Gandhi Institute of Medical Sciences Institutional Ethical Committee.

Fractionation and Preparation of sperm sample

Fractionation of human spermatozoa was done on the basis of percentage of motility by using two steps (40% and 80%) discontinuous percoll gradient technique. 40% and 80% percoll solution were prepared by mixing 100% percoll solution with Ham's F10 medium of different volume. Fresh semen samples were washed with 1X Phosphate Buffer Saline (PBS) buffer and 500µl sperm samples was layered on top of percoll gradient and incubated at 37°C for 90 min in 5% CO₂ incubator [9]. After incubation, fractionated spermatozoa were washed two times with 1X PBS buffer solution to remove percoll and prepared an aliquot of 2-3 million sperm per ml. To avoid contamination of other cells, sperm sample was treated with 50 mM Tris-Cl buffer (pH 6.8) at 6°C for 30 min prior to DNA extraction. After incubation, sperm pellet were collected by centrifugation.

Sperm mtDNA Extraction

The total DNA (nuclear DNA and mtDNA) of human spermatozoa was extracted following our standardised laboratory protocol [9]. The sperm pellet was resuspended in 0.3ml lysis buffer with proteinase-K and 10% SDS and incubated at 55°C for 2 hours. After digestion, aqueous solution was extracted with phenol:chloroform and DNA was precipitated with 3M sodium acetate and chilled absolute ethanol. After washing with 75% ethanol and dissolve in 1X TE buffer (pH 8), then DNA was ready for PCR.

Long range amplification and primer shift PCR

The long range PCR amplification of targeted sequences of mtDNA was performed with 100-150ng of template mtDNA in 50µL of total reaction volume, containing 200 mM of each dNTP, 1mM of light chain and heavy chain primers [Table/Fig-1], 2U of herculase DNA polymerase, 50mM KCL, 2mM MgCl₂, 25mM Tris(hydromethyl) methyl-3-aminopropanesulphonic acid (Agilent Technologies, USA), 1mM – mercaptoethanol, and 10mM Tris-HCL (pH 8.5). PCR was carried out for 25 cycles in two steps in first 13 cycles using the denaturation at 94°C for 1 min, annealing at 55–58°C for 1min, and primer extension at 72°C for 8 min and remaining 17 cycles denaturation and annealing condition were same but primer extension at 68°C for increasing 10 seconds to each reactions [8,9]. The amplified PCR products were then separated on a 1% ethidium

bromide containing agarose gel for 1.5 hour and observed on gel documentation system (Uvi-Tec, UK). The presence or absence mtDNA was determined using two primer pairs of Mitochondria Light Chain (MTL)-2, Mitochondria Heavy Chain (MTH)-4, same primer sets also used for confirmation of deletion finally. The primer sets MTL2-MTH4, MTL2-MTH5, MTL2-MTH6 were used for long range PCR amplification and primer shift technique was followed for the identification of existence of 7436 bp large scale deletions of mtDNA in asthenozoospermic and OAT semen samples (PCR products amplified from each of the primer pairs are shown in [Table/Fig-2]).

RESULTS

Results of percoll fractionation of sperms of patients and controls showed that low density 40% percoll fractions had contained non-motile or poor motile spermatozoa and high density 80% percoll fractions had highly motile spermatozoa.

The analysis of large scale deletions of 7436 bp mtDNA revealed that spermatozoa from 40% and 80% percoll fractions had 8 (7.2%) and 3(2.7%) mtDNA deletion respectively in infertile males using MTL2-MTH5 primer set. Out of one hundred and ten patients, 8 (7.2%) patients had showed deletions of 7436-bp (np 8637 bp to np 16073 bp). Of the eight patients showing sperm mtDNA deletions, six were asthenozoospermic (6/70; 8.5%) infertile men and two were OAT (2/40; 5%) infertile men [Table/Fig-3]. The full length PCR product of 8258-bp was observed from the wild-type mtDNA and approximately 763-bp to 822-bp bands were generated from the 7436-bp deleted sperm mtDNA. The primer shift method clearly had shown the presence of 7436-bp deletion in sperm mtDNA. The primers MTL2-MTH5 and MTL2-MTH6 were used for primer shift amplification of mtDNA and with the following primers we obtained the PCR products of 822-bp and 763-bp respectively from the 7436-bp deleted sperm mtDNA [Table/Fig-4]. Fifty samples of control fertile normozoospermic men spermatozoa were also analysed for 7436-bp mtDNA deletions from 40% and 80% percoll fractions and no such deletions were observed in both fractions.

Upon close examination of the deletions sites or breakpoints and 7436-bp deletion, we found two “12-bp direct repeats” in the junction sites from np 8637 to 8648-bp and from np 16073 to 16084-bp in

Primers	Primers Sequences	Nucleotide Positions (bp)
MTL2 (Mitochondria light chain 2)	5'-GCCCGTATTTACCCCTATAGC-3'	8251-8270
MTH4 (Mitochondria heavy chain 4)	5'-TGTAGCCGTTGAGTTGTGGT-3'	10149-10129
MTH5 (Mitochondria heavy chain 5)	5'-AGGAACCAGATGTCGGATAC-3'	16509-16490
MTH6 (Mitochondria heavy chain 6)	5'-CGAGGAGAGTAGCACTCTTG-3'	16450-16431

[Table/Fig-1]: Sequences of primers used for primer shift technique to amplify mtDNA for detection of 7436-bp deletion (Kao et al., 1998)

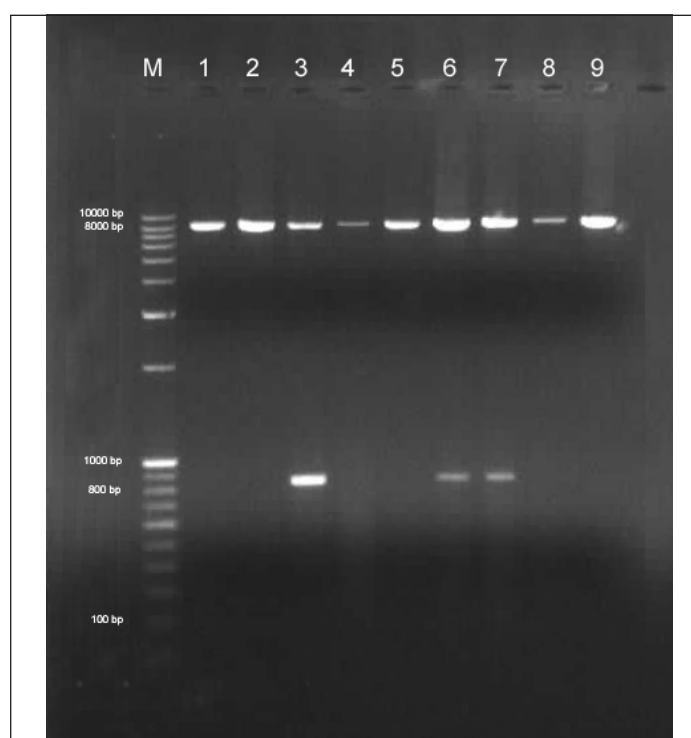
Primer Pair	Amplified region	PCR product length (normal mtDNA)	PCR product length (in deleted mtDNA)
MTL2-MTH4	8251-10149	1898-bp	--
MTL2-MTH5	8251-16509	8258-bp	822-bp
MTL2-MTH6	8251-16450	8199-bp	763-bp

[Table/Fig-2]: Nucleotide primer pairs used for the analysis of the 7436-bp deletions in the mitochondrial DNA (mtDNA) of human sperm.
MTL - Mitochondria Light Chain; MTH - Mitochondria Heavy Chain

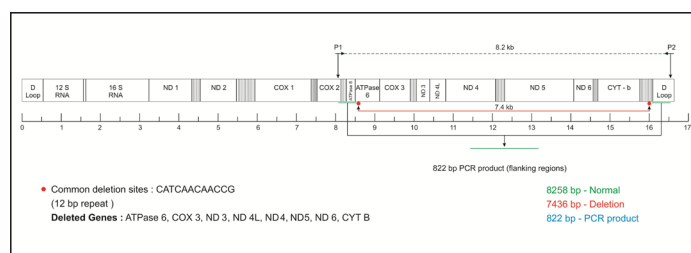
the mtDNA. Sequencing result of PCR products indicated that maximum breakpoints for 7436-bp deletions were present in the flanking regions of deleted segments. The break-points were present around the direct repeat of 12-bp (CATCAACAACCG) but rarely within the direct repeats. Thus, they generate different types of PCR products. The nucleotide sequences flanking the breakpoints of the deletions of 7436-bp of mtDNA in human spermatozoa are depicted in [Table/Fig-5].

Sample Classification	No. of Sample	Percoll Fractions		Sample-wise % of subjects with Deletions (in 40% Percoll)
		40%	80%	
		Subject-with deletion	Subject-with deletion	
Asthe	70	6	2	6/70 (8.5%)
OAT	40	2	1	2/40 (5%)
Total	110	8 (7.2%)	3 (2.7%)	8/110 (7.2%)
Normal	50	0	0	0

[Table/Fig-3]: Proportion of the 7436-bp deletion in 40%, 80% Percoll fractions of asthenozoospermia (asthe), oligoasthenoteratozoospermia (OAT) and normal fertile males.



[Table/Fig-4]: Detection of mtDNA molecules with the 7436-bp deletions by polymerase chain reaction (PCR) method. Lane M: marker 100bp-10kb and in lane 1: control DNA, by using primer pair MTL2-MTH5 we obtained the deleted and wild type mtDNA in lane 3, 6, 7. The PCR product size of the nucleotides flanking the deleted part is 822-bp and in lane 2, 4, 5, 8, 9 shown normal 8258-bp PCR product.



[Table/Fig-5]: A scheme illustrating the strategy used for the confirmation of large scale 7436-bp deletion of mtDNA, 12-bp repeat common deletions sites and deleted gene on mtDNA.

DISCUSSION

Sperm motility is important determinant of male fertility and mitochondria is essential cell organelle to generate energy for sperm

motility [14]. Non-motile spermatozoa are unable to move through the mucus layer of cervix to reach the sites of fertilization. Therefore, active motility of spermatozoa is essential for successful fertilization [15]. Several factors such as genital infections, defects in flagellar mechanism, maturation defect in the epididymis and abnormality in oxidative phosphorylation process can cause decline sperm motility and abnormal morphology of spermatozoa in asthenozoospermia and OAT infertile men [16]. Various types of sperm mitochondrial DNA deletions/ mutations have been reported in asthenozoospermia and OAT infertile men [8,9].

Analysis of our results revealed that 8.5% asthenozoospermic patients and 5% OAT patients had showed 7436-bp sperm mtDNA deletions and overall 7.2% patients had showed deletions of 7436-bp sperm mtDNA in infertile men when the sperm samples were collected from 40% percoll fractions. But, the samples from fertile normozoospermic subjects (control) did not show any such deletions. Thus, comparing the results from the subjects and controls there is a definite indication that 7436-bp deleted mtDNA in asthenozoospermia and OAT patients play a major role for sperm motility. Sequencing of PCR products indicated the break-point of large scale deletions and confirmed that the break-points were present in or around the two direct 12-bp repeats of mtDNA. In mtDNA, direct repeats are vulnerable sites for large-scale DNA deletions [17]. Studies have demonstrated that the 7436-bp large scale deletions were present in mt DNA of somatic tissues along with the mtDNA of infertile male spermatozoa [18]. Large scale 7436-bp deletions in non-motile or poor motile spermatozoa are less frequent than the "common" 4977-bp large scale deletions in mtDNA [8]. Different mechanisms have been proposed for the large scale deletions involving direct repeats present in mitochondrial genome [19,20]. Fukai and Moraes advocated that large scale deletions occurred by slipped-mispairing, illegitimate recombination, oxidative stress created by free radicals, and topoisomerase or DNA recombinase-mediated DNA breaks but still the actual mechanism of mtDNA deletions is unclear [20].

The large-scale deletions result in complete removal or truncation of some structural genes such as ATPase 6, COX-3, ND3, ND4L, ND4, ND5, ND6, CytB and eight tRNA genes of mtDNA [21]. The defective protein subunits formed by deleted or mutated mtDNA when assembled with nuclear encoded subunits lead to abnormality in respiratory enzymes [22]. Spermatozoa containing defective mtDNA not only produce ATP less efficiently, but also generate more ROS and free radicals, which may further damage mitochondria and mtDNA leading to an ultimate energy crisis and decline of motility and fertility [23]. In mitochondria, oxidative phosphorylation processes continuously produce exogenous, and endogenous free radicals with other ROS and it increases risk of mtDNA damage [21]. Generation of excess ROS and low anti-oxidant levels in the semen might cause mutation/deletions in mtDNA and impair the fertilizing capacity of spermatozoa [23]. During spermatogenesis, mtDNA deletions/mutations may occur and accumulate in the spermatids and ultimately damage respiratory function and decreases sperm motility [24]. In a study of, Kumar et al., they have reported mutations in ATPase6, ATPase8, ND2, ND3, ND4, ND5, COX3 mitochondrial genes in non-motile spermatozoa of the OAT infertile men [25]. Kao et al., showed correlation of increased ROS mediated oxidative stress with 4977-bp and 7436-bp large-scale deletions of mtDNA in non-motile spermatozoa [21]. In our previous study we also noted high frequencies of "common" large scale 4977-bp sperm mtDNA deletions in non-motile spermatozoa of asthenozoospermic and oligoasthenoteratozoospermic subjects [9]. Abasalt et al., recorded positive correlation between large scale (4.8, 7.4-kb) mtDNA deletions and abnormal human sperm motility in asthenozoospermia and OAT infertile men [26].

Results of the earlier studies [21,26] and also our previous study [9] confirmed the role of 4977-bp “common” deletions in sperm mtDNA of infertile subjects with asthenozoospermia and OAT. Some reports confirmed the association of large scale 7436-bp sperm mtDNA deletions in asthenozoospermia and OAT patients [8,12,18]. But, the frequencies of 7436-bp deletions are less than that of 4977-bp “common” deletions [26]. In the present study, we observed 7.5% deletions of 7436-bp mtDNA in patients, but there were no such deletions in fertile normozoospermic control subjects. Thus, the results indicate that large scale 7436-bp sperm mtDNA deletions play a vital role in infertile men with asthenozoospermic and oligoasthenoteratozoospermic.

LIMITATION

We have identified large scale mtDNA deletions by long-range PCR technique. Now, site specific deletions or mutations are needed to be studied by using more number of primers and sequencing of mtDNA which didn't show any deletions in asthenozoospermia and OAT subjects. These parameters are beyond our present study protocol. In future study, these limitation need to be addressed.

CONCLUSION

Non-motile abnormal sperms of asthenozoospermia and OAT cases had the mtDNA deletions of 7436-bp DNA segment which actually contains the genes responsible for oxidative phosphorylation. Normal motile sperm did not show such deletions. Oxidative phosphorylation is the source of energy for the sperm-motility and the deletions are responsible for poor sperm motility. Our result indicates that the deletions of large scale 7436-bp in mtDNA were responsible for poor sperm motility and had been associated with sperm dysfunction and male infertility.

ACKNOWLEDGEMENTS

Authors are thankful to Mr. V P Kavinesan, Mr. Mangesh Tekade and Mr. Manoj Pande for assistance in sample collection, and experimental work study. Prof. A. K. Pal and PA acknowledge ICMR, New Delhi for financial assistance. This study was supported by ICMR, New Delhi.

REFERENCE

- [1] Piomboni P, Focarelli R, Stendardi A, Ferramosca A, Zara V. The role of mitochondria in energy production for human sperm motility. *Int J Androl*. 2012;35(2):109-24.
- [2] Venkatesh S, Deecaraman M, Kumar R, Shamsi MB, Dada R. Role of reactive oxygen species in the pathogenesis of mitochondrial DNA (mtDNA) mutations in male infertility. *Indian J Med Res*. 2009;129(2):127-37.
- [3] Mishra P and Chan DC. Mitochondrial dynamic and inheritance during cell division, development and disease. *Nat Rev Mol Cell Biol*. 2014;15:634-46.
- [4] Anderson S, Bankier AT, Barrell BG, de Bruijn MHL, Coulson AR, Drouin J, et al., Sequence and organization of the human mitochondrial genome. *Nature*. 1981;290: 457-65.
- [5] Shamshi MB, Kumar R, Bhatt A, Bamezai RNK, Kumar R, Gupta NP. Mitochondrial DNA mutations in ethiopathologies of male infertility. *Indian J Urol*. 2008;24(2):150-54.
- [6] Agarwal A, Virk G, Ong C, du Plessis SS. Effect of Oxidative Stress on Male Reproduction *World J Mens Health*. 2014;32(1):1-17.
- [7] Chen T, He J, Huang Y, Zhao W. The generation of mitochondrial DNA large-scale deletions in human cells. *J Hum Genet*. 2011;56(10):689-94.
- [8] 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;52:729-36.
- [9] Ambulkar PS, Chuadhari AR, Pal AK. Association of large scale 4977-bp “common” deletions in sperm mitochondrial DNA with asthenozoospermia and oligoasthenoteratozoospermia. *J Hum Reprod Sci*. 2016;9:35-40.
- [10] Chari MG, Colagar AH, Bidmeshkipour A. A novel large-scale deletion of the mitochondrial DNA of spermatozoa of men in North Iran. *Int J Ferti Steril*. 2015;8(4):453-62.
- [11] Leremiadou F, Rodakis GC. Correlation of the 4977 bp mitochondrial DNA deletion with human sperm dysfunction. *BMC research Notes*. 2009;2:1-8
- [12] St 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;7:103-11.
- [13] World Health Organization: *Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction*. 5th edition. Cambridge University Press; 2010.
- [14] Sakthivel JP, Thangaraj G, Manonayaki S. Genetics of human male infertility. *Singapore Medical Journal*. 2009;50(4):336-47.
- [15] St John JC, Bowles EJ, Amaral A. Sperm mitochondria and fertilization. *Soc Reprod Ferti Suppl*. 2007;65:399-16.
- [16] Kumar R, Bhatt A, Bamezai RN, Shamsi MB, Kumar R, Gupta NP, et al. Necessity of nuclear and mitochondrial genome analysis prior to ART/ICSI. *Indian J Biochem Biophys*. 2007;44:437-42.
- [17] Samuels DC, Schon EA, Chinnery PF. Two direct repeats cause most human mtDNA deletions. *Trends in Genet*. 2004;20:393-98.
- [18] Colagar AH, Fatemeh K. Large scale deletions of the mitochondrial DNA in asthenoterato and oligoasthenoteratozoospermic men. *Mitochondrial DNA*. 2014;25(4):321-28.
- [19] St John JC, Jokhi RP, Barratt CL. The impact of mitochondrial genetics of male infertility. *Int J Androl*. 2005;28(2):65-73.
- [20] Fukui H, Moraes CT. Mechanisms of formation and accumulation of mitochondrial DNA deletions in aging neurons. *Hum Mol Genet*. 2008;18:1028-36.
- [21] Koa SH, Chao HT, Wei YH. Multiple deletions of mitochondrial DNA are associated with the decline of motility and fertility of human spermatozoa. *Mol Hum Reprod*. 1998;4(7):657-66.
- [22] Gashti NG, Salehi Z, Madani AH, Dalivandan ST. 4977-bp mitochondrial DNA deletions in infertile patients with varicocele. *Andrologia*. 2014;46(3):258-62.
- [23] Chen SJ, Allam JP, Duan YG, Haidl G. Influence of reactive oxygen species on human sperm functions and fertilizing capacity including therapeutical approaches. *Arch Gynecol Obstet*. 2013;288(1):191-99.
- [24] Rajender S, Rahul P, Mahadi AA. Mitochondria, Spermatogenesis and male infertility. *Mitochondrion*. 2010;10(5):419-28.
- [25] Kumar R, Venkatesh S, Kumar M, Tanwar M, Shamsi MB, Kumar R, et al. Oxidative stress and sperm mitochondrial DNA mutation in idiopathic oligoasthenozoospermic men. *Indi J Biochem Biophys*. 2009;46:172-77.
- [26] Abasalt HC, Gholamali JS, Maryam GC. Lipid peroxidation and large-scale deletions of mitochondrial DNA in asthenoteratozoospermic patients. *Indian J Bioche Biophys*. 2013;50:492-99.

PARTICULARS OF CONTRIBUTORS:

1. Senior Research Fellow and Department of Anatomy, Human Genetic Division, Mahatma Gandhi Institute of Medical Sciences, Sevagram, Wardha, Maharashtra, India.
2. Associate Professor, Department of Anatomy, Mahatma Gandhi Institute of Medical Sciences, Sevagram, Wardha, Maharashtra, India.
3. Professor and Head Department of Physiology, Mahatma Gandhi Institute of Medical Sciences, Sevagram, Wardha, Maharashtra, India.
4. Associate Professor, Department of Anatomy, Mahatma Gandhi Institute of Medical Sciences, Sevagram, Wardha, Maharashtra, India.
5. Professor, Department of Anatomy, Mahatma Gandhi Institute of Medical Sciences, Sevagram, Wardha, Maharashtra, India.
6. Professor and Head, Department of Anatomy, Mahatma Gandhi Institute of Medical Sciences, Sevagram, Wardha, Maharashtra, India.
7. Professor, Department of Anatomy, Human Genetic Division, (Cytogenetics and Molecular Genetics), Mahatma Gandhi Institute of Medical Sciences, Sevagram, Wardha, Maharashtra, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:

Asoke K. Pal,
Professor, Department of Anatomy, Human Genetics Division, Mahatma Gandhi Institute of Medical Sciences,
Sevagram, Wardha -442102, Maharashtra, India.
E-mail: asokepal@yahoo.com

FINANCIAL OR OTHER COMPETING INTERESTS: None.

Date of Submission: Jun 29, 2016

Date of Peer Review: Jul 22, 2016

Date of Acceptance: Sep 03, 2016

Date of Publishing: Nov 01, 2016