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RESEARCH ARTICLE

NOVEL MUTATIONS (8899C>T AND 8907C>T) OF THE MITOCHONDRIAL ATPASE 6 GENE IN IRAQI ASTHENOZOOSPERMIC PATIENTS

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ABSTRACT

Proposed causes of male infertility include sperm motility disturbances, Y chromosome microdeletions, chromosomal abnormalities, single gene mutations, and sperm mitochondrial DNA (mtDNA) rearrangements. To investigate the etiology of decreased sperm fertility and motility of sperm and to develop an appropriate therapeutic strategy, the molecular basis of these defects must be elucidated. In this study, we aimed to reveal the relationships between the genetic factors including sperm mtDNA mutations as candidate factors for male infertility. 56 Iraqi men with a history of asthenozoospermia and 10 fertile men were recruited to the study. Mitochondrial ATPase6 gene was amplified by PCR and then analyzed by direct sequencing. Two novel nucleotide substitutions were identified in the examined mitochondrial gene (ATPase 6) in both subjects as well as in progressive and non-progressive sperms, all of which are statistically significant. Two silent novel substitutions 8899 C>T and 8907C>T in ATPase6 gene that occur without change in the amino acids of protein. These heteroplasmic silent mutations(8899C>T and 8907C>T) were observed with a high frequency in 0-5% group of sperm motility (according to WHO criteria) which were 15%(10)and 15%(10), respectively, while these frequencies were less in the rest sperm motility groups covered in this study, also, the lowest frequencies were in the normozoospermic subjects. The highest frequencies of these mutations were in the non-progressive motile sperms cells fractions that were isolated from 40% gradient in 0-5% group of sperm motilitywhich were13.5%(9)and 12%(8) for 8899C>T and 8907C>T mutations, respectively, rather than in progressive motile sperms cells that were isolated from 80% gradient fraction. The present study clearly demonstrated that mutation of mtDNA does play important roles in the diminution of fertility and decline of various motility parameters that are important determinants of male fertility as well as it approved that the mtDNA novel mutation occurrence is possible with high frequency.

INTRODUCTION

Infertility is a major medical and social problem worldwide, which impacts people both medically and psychosocially (Fisher and Hammarberg, 2012). According to the World Health Organization, 60–80 million couples suffer from infertility worldwide (Rutstein *et al*., 2004). A male partner factor contributes to 40% of cases of infertility (Alam, 2009). Male infertility is associated with asthenozoospermia or oligoasthenozoospermia and has been reported in patients suffering from typical mtDNA diseases, involving point mutations or multiple deletions of mtDNA (Sampson *et al.,* 2001). Sperms have been shown to be particularly prone to developing deletions of mtDNA and these deletions are associated with the decline of sperm motility and then fertility. However, it has been shown that mtDNA point mutations, mtDNA single nucleotide polymorphisms (SNPs) and mtDNA haplogroups can greatly influence semen quality (Kumar and Sangeetha, 2009). Asthenozoospermia, a disorder of sperm motility, is a cause of human male infertility and is implicated in 19% of infertile cases (Curi *et al*., 2003). Isolated asthenozoospermia is found in 24% of infertile men (Luconi *et al*., 2006), which may be caused by sperm dysfunction, prolonged periods of sexual abstinence, partial blockage of seminal tract, varicocele, infection or genetic factors (Gdoura *et al*., 2007; Pasqualotto *et al*., 2008a,b; Martini *et al*., 2010; Jaiswal *et al*., 2012). However, some cases of asthenozoospermia could be idiopathic; namely, no definitive etiology is identifiable by using routine medical tests (Ortega *et al*., 2011). Mitochondrial DNA mutates at a rate of 10-20 times higher than nuclear DNA due to asexual method of replication, a very basic repair mechanism, lack of protective histones and increased proximity to the site of free radical generation. There are 70-80 mitochondria in the midpiece of mammalian sperm. It is long established that there is one copy of mtDNA in each mitochondrion in the midpiece of mammalian sperm (Kumar *et al*., 2009 ; Venkatesh *et al*., 2009). Spermatozoa exist in a

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state of oxygen paradox and have higher energy requirement to support theirmovement after ejaculation. Thus, random attacks on the nacked mtDNA of sperms by reactive oxygen species (ROS) or free radicals causes oxidative damage or mutation to the mitochondrial genome, which leads to infertility (Venkatesh *et al*., 2009; Venkatesh *et al.,* 2009). To our knowledge, there is no available data about the relationship between mtDNA changes and asthenozoospermia were previously identified in Iraq. The aims of this study was to screen some mutations in mtDNA ATPase 6 gene which influence the sperm motility.

MATERIALS AND METHODS

Subjects

Fifty-six Iraqi men with asthenozoospermia who attended the physician in Kamal-Samarae hospital for *IVF* and treatment of infertility in Bagdad, Iraq as well as 10 Iraqi healthy men who served as a control were selected. Sampling was carried out in Baghdad, Iraq during a period from June 2012 to October 2012.

Inclusion and exclusion criteria

Inclusion criteria

Male partner with at least one year infertility. A minimum of t wo semen analysis at an interval of 6 weeks showingastheno- zoospermia. Total sperm count is $40x10^6$ /mlspermatozoa or more. Sperm motility is fewer than 50% spermatozoa with forward progression (grades A and B or fewer than 25% spermatozoa with grade A movement according to WHO criteria (WHO, 2010).

Exclusion criteria

Varicocele, accessory sex gland infection, testicular maldescen t, previous reproductive organ surgery, and sexually transmitted diseases had been excluded. Patients categorized under azoospermia had been excluded. Past history of mumps, orchitis, trauma, addictions, and acute febrile illnes s had been taken into account. Diabetes, thyroid disorders, tuberculosis, vascular diseases, and any long standing infection had also been taken into consideration.

Semen evaluation

Semen sample was collected in a sterile plastic container after sexual abstinence of 3-5 days and incubated for 30 minutes at 37°C to liquefy. Semen analysis of patients and healthy had been carried out by using a Computer Assisted Semen Analysis (CASA) as recommended standards of semen examination by WHO (2010), which included the measurement of spermatozoon concentration, morphology, and motility. Four types of motility were assessed and classified as follows: a) rapid progressive or linear motility; b) slow progressive or curvilinear motility; c) not progressive or in loco motility; d) lack of motility.

Discontinuous Density Gradient Centrifugation (Percoll)

Each individual asthenozoospermic and control samples were divided into two fractions i.e. fast progressive and slow motile sperm populations (non- progressive) by using discontinuous density gradient PureSperm® 40 and PureSperm® 80, (Nidacon, Flöjelbergsgatan Mölndal, Sweden) was used. Firstly aspiration 2ml by micropipette with sterile tip from 20 ml container of the 80% PureSperm® gradient solution was placed in the bottom of new sterile Conical Centrifuge Tube. Subsequently aspiration 2ml by micropipette with sterile tip of 20 ml container of the 40% gradient was layered on top slowly and carefully, finally two gradient layers were formed, after that 2ml of the fresh collected liquefied and examined semen, slowly and carefully was poured on top of the 40% layer and the tube was centrifuged with for 20 min at 300xg by using (Eppendorf , Germany).After centrifugation different layers were formed from top to bottom (first seminal plasma, second Immotile /dead sperms, debris, leukocytes, epithelial cells and bacteria, third contained immature and senescent sperm, fourth contained non progressive sperm cells and the last is a pellet which contained highly progressive motile sperm cells. The non- progressive sperms cells were retrieved from the interface between the 40% and 80% gradient while the fast progressive cells accumulated as a pellet at the bottom of the tube.

DNA Isolation

One milliliter sperms cells suspension was transferred to microtube 1.5ml and DNA was extracted from spermatozoal cells according to manufacturer protocol of kit Diatom^{TM}DNA Prep 100 (Isogene, Russia),which is based on use of lysic reagent with guanidinthiocyanate. The DNA concentration and purity were estimated by using Nanodrop (BIONEER, Korea) at 260/280 nm wave length.

DNA Amplification

From sperm of the 56 infertile and men with asthenozoospermi a as well as 10 fertile men, mitochondrial DNA gene (ATPase 6 gene) were amplified andsequenced. Primer sequences for the above gene was designed using the online program Primer3Plus web (http://www.bioinformatics.nl/cgibin/primer 3plus/primer3plus.cgi/) as shown in (Table 1). PCR was carried out in a 0.2-mL thin-wall tube using 5.0 ng of DNA, 0.5 μM of each primer, 200 μM of dNTPs, 1× PCR Phusion HF Buffer containing 5 mM $MgCl_2$,and 0.02 U of Phusion Hot Start II DNA Polymerase (New England Biolab).

Table 1. Forward and reverse primers targeting mitochondrial ATPase 6 gene used in this study

primer	sequences	Primer site	Band size (bp)
ONP Forward	5- CTACGGTCAATGCTCTGAAA-3	8161-8180	1078
ONP185-Reverse	5-TACTATATGATAGGCATGTGA -3	9239-9219	

DNA Electrophoresis

The PCR amplified products were run by electrophoresis on gel (1.5%) by aspirating 4μl of PCR amplified products and mixed gently with 1*ul* loading dye (Thermoscientific 6x DNA Loading Dye) as well as to positive control. A1*ul* of 100 *bp* DNA ladder (GeneRuler, Fermentas) was used as the

molecular standard in order to confirm the amplified PCR product as revealed in (Figure 2).

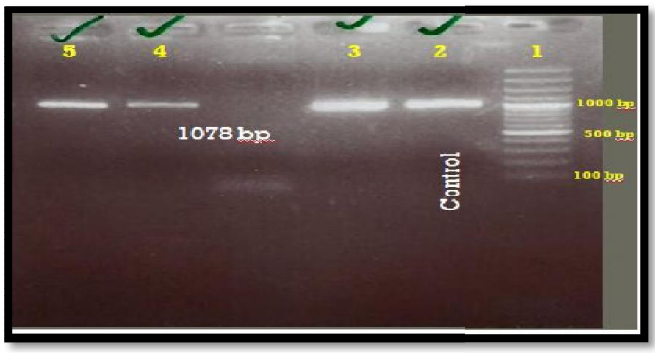

Figure 2. Representative PCR products (1078bp) stained with Rima sight DNA stain and visualized under UV light .Lane 1= 100 bp DNA ladder (GeneRuler, Fermentas), *lane 2*= Wild Control, *lanes 3,4and 5*= PCR products 1078bp

Sequencing Reaction and Electrophoresis

The PCR products of ATPase6 was directly determined by automated sequencing in a 3700 ABI machine (Applied Biosystems, Foster City, CA, United States). The sequencing master mixes contained the ONP 185 reverse primer. Cycling conditions were: heat activation at 96 ^{0}C for 1min, followed by 25 cycles at 96 ^{0}C for 10sec, 50 ^{0}C for 5sec, and 60 ^{0}C for 4 min. Sequencing reaction clean-up was performed. PCR products were electrophoresed using the AB 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, United States).

Sequences analysis of ATPase6 gene

Sequencing data were analyzed *via* Variant Reporter™ Software version 1.0 (Applied Biosystems, Foster City, CA,United States) and compared with the reference sequence from Genbank (accession No. NC_012920) to indicate possible polymorphisms. The corresponding changes of amino acid with missense and silent polymorphisms were excepted from MITOMAP (www.mitomap.org). For polymorphism not recorded in MITOMAP, HmtDB (http://www.hmtdb.uniba .it: 8080/ hmdb/) was applied.

Statistical Analysis

The statistical analysis of the data was performed using Graph pad Prism 5.0 Software (La Jolla, CA, USA).The Pearson's chi-squared test (χ^2) was used to compare patients subjects with control group and the value of $P<0.05$ were considered significant.

RESULTS AND DISCUSSION

The mitochondrial ATPase6 gene was amplified by using conventional PCR and PCR products were sequenced from sperm cells of the infertile men. Sequence was analyzed by using Variant Reporter® Software version 1.0 (Applied Biosystems®,USA, CA) and variation is being recorded by aligning mtDNA sequences to the revised Cambridge reference sequence (rCRS) and the alignment was carried out through (www.mitomap.org andhttp://mitomap.org/bin/ view.pl/MITOMASTER /WebHome). Comparison of the

Table 2. Conventional PCR cycles for amplification conditions

step	Temperature (C)	time	cycles
Initial denaturation	94	4 min	1
denaturation	94	50 sec	32
annealing	72	55 sec	
extension	72	45 sec	
Final extension	72	10 min hold	1
	4		

Table 3. Novel silent 8899C>T mutationfrequency in mitochondrial ATPase 6 gene of asthenozoospermic versus normospermic subjects

	Sperm motility (%)						
WHO	Control N=10	0-5 N=10	6-10 N=14	11-15 N=12	16-20 N=10	21-25 N=10	Mean± SE
Percoll							
40 %	0%(0)	13.5%(9)	7.5%(5)	7.5%(5)	1.5%(1)	0%(0)	3.33±1.5
80 %	0%(0)	1.5%(1)	0%(0)	0%(0)	0%(0)	0%(0)	0.17±0.17
P value							0.05 *
Total	0%(0)	15%(10)	7.5%(5)	7.5%(5)	1.5%(1)	0%(0)	
Type (Effect)			Substitution (Silent)				
A.A. change			Leu to Leu				
Mitomap reference			Novel				

SE mean standard error. *mean significant at 0.05 level.

Table 4. Novel silent 8907C>T mutationfrequency in mitochondrial ATPase 6 gene of asthenozoospermic versus normospermic subjects

	Sperm motility (%)						
WHO	Control N=10	0-5 N=10	6-10 N=14	11-15 N=12	16-20 N=10	21-25 N=10	Mean± SE
Percoll							
40 %	3%(2)	12%(8)	10.5%(6)	4.5%(3)	6%(4)	3%(2)	4.17±0.98
80 %	0%(0)	3%(2)	6%(1)	1.5%(1)	0%(0)	0%(0)	0.67±0.33
P value							0.007**
Total	3%(2)	15%(10)	10.5%(7)	6%(4)	6%(4)	3%(2)	
Type (Effect)			Substitution (Silent)				
A.A. change			His to His				
Mitomap reference			Novel				

SE mean standard error. **mean significant at 0.01 level.

above gene sequence with a reference sequence revealed there are two novel nucleotides substitutions in ATPase6 gene of sperm mtDNA, where these mutations occurred among asthenozoospermic which were included 56 infertile samples and 10 normal men (a total of 66 samples) and these mutations greatly distributed with different frequencies among the asthenozoopermic cases rather than normospermic subjects. By the results obtained in the present study it has been shown that the highest frequency of novel heteroplasmic silent mutations was observed in 0-5% group of sperm motility (according to WHO) were15% (10) and 15% (10) in ATPase6 gene 8899C>T and 8907C>T, respectively. Whilst mutations were less frequency in the rest of the sperm motility groups covered in this study, also were at the lowest frequency in the normozoospermic subjecst ($p<0.05$) as listed in (Table 2). On the other hand, the results of this study showed that the highest frequency of these mutations in the non- progressive motile sperms cells fractions that were isolated from 40% gradient8899C>T and 8907C>T were 13.5%(9) and 12%(8), respectively, rather than in progressive motile sperms cells that were isolated from 80% gradient fraction as shown in (Tables 3 and 4). Results in the present study indicate that the high frequency incidence of the mitochondrial mutations in ATPase 6 gene in this study that occurred among asthenozoospermic subjects rather than in control subjects came agreed with the results that found by Kumar *et al*. (2009) who found a significant nucleotide (nt) change in the gene ATPase8 gene (nt 8394) of infertile subjects compared to the control subjects. Also, Kumar *et al.* (2009) observed that there is asignificant nucleotide changes in the mitochondrial genes (ATPase 6, ATPase 8, ND2, ND3, ND4 and ND5) in the semen of the infertile men which were came agreed with the result of this study. Palanichamy and Zhang (2011) foundt a significantly higher nucleotide changes in the mtDNA genes: COII, ATPase6, ATPase8, ND2, ND3, ND4and ND5 in spermatozoa from populations of Indian infertile men.

In addition, Barbhuiya *et al.* (2012) found that the incidence of mtDNA mutations (8557G>C) in ATPase8 gene occurred in asthenoteratozoo-spermia. A latest study by Baklouti-Gargouri *et al.(*2013) using mtDNA sequencing analysis they were detected a novel heteroplasmic missense mitochondrial mutation (9387 G>A) in COXIII gene (8.8%) of 3 asthenozoospermic patients that was not found in any of normospermic infertile and fertile men. This mutation substituting the valine to methionine in a conserved amino acid. Generally, antioxidants are present in cell to scavenge the free radicals generated in the environment and thus prevent the damage caused to the cell. But, in spermatozoa, due to shedding of cytoplasm during spermiogenesis, the absence of enzymatic antioxidants is usually compensated by the seminal constituents of different origins (Yeung *et al.,* 1998). The antioxidants enzymes (catalase and Gpx) were found to be significantly lower in cases when compared to the fertile men (Kumar *et al.,* 2009). As mtDNA are source of production of ATP through OXPHOS, it's also site of reactive oxygen species production and first site of ROS-induced damage (Kumar *et al.,* 2009). Seminal oxygen species can damage sperm by different mechanisms, and is a common pathology seen in two-thirds of infertile cases (Whittington *et al.,* 1999; Tremellen, 2008). Agarwal *et al.* (2006) recently reported that high levels of ROS could be an independent marker of male-factor infertility. Wang *et al.* (2009) detected a 3.3-fold increase in the level of ROS in asthenozoospermic semen. Moreover, sperms with mtDNA mutation may also have high nuclear DNA fragmentation, which is a promutagenic change that could generate mutation in the offspring (Aitken and Krausz, 2001). Thus, screening of mtDNA is necessary to understand the etiology of motility disorders in oligoasthenozoospermic men. Such cases which harbor OS-induced mtDNA mutations have a better diagnostic (Marchington *et al.,* 2002). There are three probable mechanisms of mutagenesis leading to generation of mtDNA nucleotide substitutions: (1) deamination of DNA bases; (2) tautomeric migrations of protons in nitrous bases; and (3) hydrolysis of glycoside link between DNA bases and carbohydrate residue on the background of free radical damage of the mitochondrial DNA polymerase gamma. By means of quanto-chemical calculations, it was shown that the most substantiated mechanism of mutation generation is hydrolysis of N-glycoside link. This mechanism is suggestive to be more prominent on the H-strand, which remains to be single-stranded for a long time during the mtDNA replication (Kornienko and Maliarchuk, 2005). Oxygen species may also damage or alter the DNA sequence of mitochondrial genome, thus decreasing the production of ATP and further increasing the leakage of free radicals through electron transport chain (Venkatesh *et al.,* 2009). As during spermatogenesis, there is an increase in mtDNA copy per cell (Díez-Sánchez *et al.,* 2003) defect in mitochondrial genome may increase the free radical production in the sperms, as compared to other somatic cells. Moreover, increased Malondialdehyd (MDA) in asthenozoospermic people may damage or alter DNA through oxidation or covalent binding (Ernster, 1993).

Conclusion

The present study clearly demonstrated that mutation of mtDNA does playimportant roles in the diminution of fertility and decline of various motility parameters that are important determinants of male fertility as well as it approved that the mtDNA novel mutation occurrence is possible with high frequency.

REFERENCES

Agarwal, A.; Sharma, R.K.; Nallella, K.P.; Thomas, A.J.; Jr, Alvarez, J.G. and Sikka, S.C.2006. Reactive oxygen species as an independent marker of male factor infertility. Fertil. Steril. 86: 878–85.

Aitken, R.J. and Krausz, C. 2001. Oxidative stress, DNA dama ge and the Y chr omosome. Reproduction. 122(4): 497-506.

Alam, N. (2009): Male factor infertility basics revisited. J Coll Physicians Surg. Pakistan.19:205 - 206.

Barbhuiya, P.N.; Gogoi, A.; Ahmed, G. and Mahanta, R. 2012. A study on genetic aspects of male infertility in North-east Indian population, India. BMC Proceedings. 6(Suppl 6): 3.

Baklouti-Gargouri, S.; Ghorbel, M.; Ben Mahmoud, A.; Mkaouar-Rebai, E.; Cherif, M.; Chakroun, N.; Sellami, A.; Fakhfakh, F. and Ammar Keskes, L. 2013.

Mitochondrial DNA mutations and polymorphisms in asthenospermic infertile men. *Mol Biol Rep*. 40(8):4705-12.

Curi, S.M.; Ariagno, J.I.; Chenlo, P.H.; Mendeluk, G.R.; Pugliese, M.N.; Sardi,L.M.; Repetto, H.E. and Blanco, AM. 2003. Asthenozoospermia:analysis of a large population Arch Androl.49:343-49.

Dı´ez-Sa´nchez, C.; Ruiz-Pesini, E.; Lapen˜a, AC.; Montoya, J.; Pe´rez-Martos, A.; Enrı´quez, JA. and. Lo´pez-Pe´rez. M.J. 2003. Mitochondrial DNA Content of Human Spermatozoa. *Biology of reproduction.*68:180-185.

Ernster, L. 1993. Lipid peroxidation in biological membranes: mechanisms and implications. In: Active Oxygen, Lipid Peroxides and Antioxidants (Yagi K, ed),1-39, CRC Press, Boca Raton.

Fisher, J.R. and Hammarberg, K. 2012. Psychological and social aspects of infertility in men: an overview of the evidence and implications for psychologically informed clinical care and future research. *Asian J Androl*. 14:121-129.

Gdoura, R.; Kchaou, W.; Chaari, C.; Znazen, A.; Keskes, L.; Rebai T. and Hammami A. 2007. Ureaplasma urealyticum, Ureaplasma parvum, Mycoplasma hominis and Mycoplasma genitalium infections and semen quality of infertile men.

Jaiswal, D.; Sah, R.; Agrawal, N.K.; Dwivedi, U.S.; Trivedi, S. and Singh, K. 2012. Combined effect of GSTT1 and GSTM1 polymorphisms on human male infertility in north Indian population.*Reprod Sci*. (19):312-316

Kornienko, I.V. and Maliarcchuk, B.A. 2005. Aanalysis of mutation mechanismsin human mitochondrial DNA.Mol. Biol.(Mosk). 39(5):869-77.

Kumar, R.; Venkatesh, S.; and Kumar M. 2009. Oxidative stress and sperm mitochondrial DNA mutation in idiopathic oligoasthenozoospermic men. *Indian Biochem Biophys*. 46:172-7.

Luconi, M.; Forti, G. and Baldi, E. 2006. Pathophysiology of sperm motility. *Front Biosci* 11:1433 - 1447.

Marchington, D R.; Scott, Brown, M S.; Lamb, V K.; van Golde, R J.; Kremer, J A.; uerlings J H.; Mariman, E C.; Balen, A H. and Poulton, J.2002. Noevidence for paternal mtDNA transmission to offspring or extra-embryonic tissues after ICSI. Mol Hum Reprod .8, 1046-1049.

Margulis, L. 1981. Symbiosis in cell evolution. New York: W.H. Freeman, 452. [Biology Department, University of Massachusetts. Amherst. MA]

Martini, AC.; Tissera, A.; Estofán, D.; Molina, RI.; Mangeaud, A.; de Cuneo, MFand Ruiz, RD. 2010. Overweight and seminal quality: a study of 794 patients. Fertil Steril. 94(5):1739-43.

Ortega C, Verheyen G, Raick D, Camus, M.; Devroey, P, and Tournaye, H. 2011. Absoluteasthenozoospermia and ICSI: What are the options? Hum Reprod Update 17:684–692,

Palanichamy, M. G. and Zhang, YP. 2011. Identifying potential pitfalls in interpreting mitochondrial DNA mutations of male infertility cases Indian *J Med Res*. 134(4): 447–451.

Pasqualotto, FF.; Sharma, RK.; Pasqualotto, EB. And Agarwal, A. 2008a. Poor semen quality and ROS-TAC scores in patients with idiopathicinfertility. Uro;81(3):263-70.

Pasqualotto, FF.; Sundaram, A.; Sharma, RK.; Borges, E Jr.; Pasqualotto, EB. And Agarwal, A. 2008b. Semen quality and oxidative stress scores in fertile and infertile patients with varicocele. Fertil Steril. 89:602-607.

Rutstein, S.O. and Shah, I.H. 2004. Infecundity, Infertility, and Childlessnessin Developing Countries. ORC Macro. MEASUREDHS+ (Programme) and World Health Organization .Calverton, MD, Geneva, Switzerland: OR C Macro World Health Organization.

Tremellen, K. 2008. Oxidative stress and male infertility a clinical perspective. Hum Reprod. Update.14: 243–58.

Venkatesh,S.; Deecaraman, M.; Kumar, R.; Shamsi,M.B. and Dada, R. 2009. Role of reactive oxygen species in the pathogenesis of mitochondrial DNA (mtDNA) mutations in male infertility. *Indian J Med Res*. 129(2):127-37.

Wang, J.; Wang, J; Zhang, H.; Shi, H.; Ma, Zhao, H.; Lin, B. and Li, R. 2009. Proteomic analysis of seminal plasma from asthenozoospermia patients reveals proteins that affect oxidative stress responses and semen quality. *Asian J Androl*.11: 484–491.

Whittington, K. and Ford, W.C. 1999. Relative contribution of leukocytes and of spermatozoa to reactive oxygen species production in human sperm suspensions. *Int J Androl*. 22: 229-235.

World Health Organization.WHO 2010. Laboratory Manual f or the Examination and Processing of HumanSemen. 5th ed. Cambridge, UK: Cambridge University Press.

Yeung, C. H.; Bals-Pratsch, M.; Knuth, U. A.; and Nieschlag, E. 1988. Investigation of the cause of low sperm motility in asthenozoospermic patients by multiple quantitative tests. *International Journal of Andrology*.11: 289–99.

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