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RESEARCH ARTICLE

ALTERATION OF THE COPY NUMBER OF MITOCHONDRIAL *COXI* GENE IN THE SPERMS OF IRAQI ASTHENOZOOSPERMIC PATIENTS

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ABSTRACT

The present study was conducted to estimate the copy number of mitochondrial *COXI* gene in the sperm cells from Iraqi asthenozoospermic patients by using q PCR. Cases were established of Asthenozoospermia from Kamal AL.Samraee Hospital for Infertility and *In Vitro* Fertilization in Baghdad-Iraq. The relative quantification of mtDNA copy number was performed for 28 sperm extracts (28 fractions 80 % layers and 28 fractions 40% layers) obtained from 28ejaculates, 16 samples were from asthenozoospermic patients and 12 samples were from control subjects. The content of mtDNA per haploid mitochondrial genome in sperm cell was significantly different ($p \leq 0.05$) between non- progressive motile sperm cells and mtDNA content corresponded to the progressive sperm (119.9 ± 26.89 and 58.99 ± 22.68, respectively). Increasing of relative mtDNA copy number of defective sperm fraction and normospermic fractions were 74.2-271.3 and 1.9-92.8/copy per cell, respectively. No significant difference between progressive motile sperms of infertile subjects and progressive motile sperms of control subjects that were isolated from 80% percoll layer as well as to non progressive motile sperms of both groups that were separated from 40% Percoll fraction. There is a highly significant difference ($p \leq 0.05$) between infertile subject and control subject on average 128.4 ± 14.76 and 60.90 ± 6.83 copy per cell.

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). Sperm motility is important for movement from the vagina to the Fallopian tubes, for penetration of the cumulus oophorus, and for processes involved in fertilization (Ortega *et al.* 2011). Asthenozoospermia is a common cause of male infertility characterized by reduced forward motility or an absence of sperm motility in fresh ejaculate (Ortega *et al.,* 2011; Jodar *et al.,* 2012). Mitochondria are factories for the production of energy, whose oxidative phosphorylation system (OXPHOS) drives sperm motility (Pascual *et al.,* 1996, Ruiz-Pesini *et al.,* 1998). Most of the enzymatic complexes in the OXPHOS are partially encoded by mitochondrial DNA (mtDNA). Therefore, the genetic alteration of mtDNA will directly impact on the function of the OXPHOS, and may ultimately cause fertility problems (Nakada *et al.,* 2006). The copy number of mtDNA (i.e., the number of mtDNA per spermatozoon) is one of the major mitochondrial genetic features, and plays a critical role in mtDNA-mediated pathological pathways (Díez-Sánchez *et al.,* 2003, Liu *et al.,* 2006). For example, in infertile men with abnormal sperm quality, mtDNA content was found to be significantly increased, and could serve as a marker for spermatogenic dysfunction (Song and Lewis 2008). Besides mtDNA copy number, the change of mtDNA integrity is another important factor causing asthenospermia, or poor sperm motility, in infertile men. Patients with low sperm motility were observed to have remarkably compromised mtDNA integrity (Koa *et al.,* 1995). In this study, we investigated the mtDNA copy number of mtDNA (*COXI* gene) and nDNA (*β-actin* gene), respectively using relative qPCR in semen samples taken from Iraqi asthenozoospermic patients according to the criteria of the World health Organization (2010).

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MATERIALS AND METHODS

Subjects

Semen samples were collected from 16 asthenozoospermic men and 12 normal subjects (according to fertility parameters). Cases were established of Asthenozoospermia from Kamal AL.Samraee Hospital for Infertility and *In Vitro* Fertilization in Baghdad, Iraq. This study was conducted during a period from April 2012 to July 2013.

Inclusion and exclusion criteria

Inclusion criteria: Total sperm concentration is $40x10^6$ sperm atozoa / ml or more. Rapid linear forward progressive motility for grade A less than 25% or less than 50% for grades A+B according to WHO criteria (WHO, 2010).

Exclusion criteria: Patient has a history with pathological condition that related reproductive system like infectious diseases (mumps, orchitis and sexual transmitted diseases, STD), metabolic disorders (Diabetes) and patients categorized under azoospermia.

Ethics

All semen samples were taken from participants with informed consent following the ethical guidelines of the ethics committee of our institute and hospital.

Semen Evaluation

Semen samples were collected in a sterile plastic containers 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.

Experimental Design

Sixteen Asthenozoospermic samples and 12 control were collected according to WHO (2010) criteria, then each individual semen sample was divided into two fractions i.e. rapid progressive and non progressive sperm populations by discontinuous gradient centrifugation (Percoll). All samples were fractionated 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 placed on top of the 40% layer and the tube was centrifuged with for 20 min at 300xg by using (Eppendorf, Germany). After centrifugation with the different layers were formed from top to bottom (first seminal plasma, second Immotile /dead sperms, debris, leukocytes, epithelial cells and bacteria and last third contained immature and senescent sperm, fourth contained slow motile sperm cells and the last is a pellet which contained highly progressive motile sperm cells, and carefully removed from top side of tube without any disturbances and mixing. The slow and low motile 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 this technique done under highly aseptic conditions.

DNA Isolation

DNA isolation was carried out under highly aseptic technique and extraction of DNA from spermatozoal cells was done by using DNA extraction isolation according to manufacturer protocol of kit Diatom™ DNA Prep 100 (Isogene, Russia). Which is based on use of lysic reagent with guanidinthiocyanate.

Relative qPCR of mtDNA Copy Number

The relative mtDNA content was measured by a real-time PCR on cytochrome c oxidase I (*COX I*) gene and normalized by simultaneous measurement of nuclear DNA encoded *ß-actin* gene. QPCR was carried out using of Corrbet Real-Time PCR System (Corrbet, Australia). A master mix was prepared for mtDNA and nDNA separately with a total volume 15µl reaction in different tubes (72 x 0.1 ml Strip Tubes) containing SYBR® Premix 1X, 0.1 µM each of the forward and reverse primers, and 5*ng* of DNA sample for mtDNA and 5*ng* for nDNA were diluted by TBE buffer and measured by nanodrop (BIONEER, Korea) at 260/280 *nm* wave length. A non template control (NTC) was prepared which contains all master mix components except template DNA as shown in (Table 1) and all steps of this process were carried out under cooling conditions by using Loading Block (72 x 0.1 ml Strip Tubes).

Table 1. QPCR Master Mix Components for target mitochondrial COXI gene and reference nuclear ß-actin gene

Reagent	Volume (µl)	Final concentration
SYBR® Premix Ex Taq II (2X) (Tli RNaseH Plus), Bulk	7.5	1X
Forward Primer	1	0.1 µM
Reverse Primer	1	0.1 µM
Template DNA	1	5ng
dH2O	4.5	
Total	15	

Table 2. Primers sequences for target MT-COXI gene and reference nuclear ß-actin

Gene	Primers	Sequence	Site	Length
Mitochondrial COXI gene	Forward	5-TTCGCCGACCGT TGACTATTCTCT-3	5907-5930	24nt
	Reverse	5-AAGATTATTACA AATGCATGGG-3	6103-6082	22nt
Nuclear β-actin gene	Forward	5-ACCCACACTGT GCCCATCTAC-3	1997-2017	21nt
	Reverse	5-TCGGTGAGGAT CTTCATGA GGTA-3	2103-2081	23nt

Solid aluminium block then all tube were mixed and spin gently then transferred to 72 wells rotor disc. The PCR conditions were set up 95°C for 90sec, followed by 40 cycles of 95°C for 15sec, 58°C for 20sec and 72°C for

20sec.The threshold cycle number (Ct) values of the ß-actin gene and the mitochondrial COXI gene were determined automatically by machine. Changes in relative gene expression between groups were calculated by using the $2^{-\Delta\Delta CT}$ method with normalization to ß-actin. Specific primers for ß-actin were designed using the Primer Express Program (Applied Biosystems, Foster City, CA) and the mtDNA COXI primers were designed using the online Primer3Plus web (http://www.bioinformatics.nl/cgibin/primer 3plus/primer 3plus.cgi/)as presented in (Table 2). Each measurement was carried out in technical triplicate and normalized by a control DNA sample.

RESULTS AND DISCUSSION

In order to compare the mtDNA content of normal and abnormal sperm, we eliminated round cells using a discontinuous density gradient centrifugation capable of selecting only motile sperm. The absence of round cells in sperm preparations was checked by light microscopy. The accurate quantification of mtDNA depends closely on the high recovery of mtDNA templates (Reynier *et al*., 2001). The mitochondrial COXI /nuclear *β*-actin ratio, which expressing of the average copy number per haploid genome or per sperm, was determined for 28 sperm extracts (28 fractions 80 % layers and 28 fractions 40%layers) obtained from the 28 ejaculates, 16 subjects from asthenzoospermic patients and 12 samples from control subjects that their progressive motility class A> 25% and class A+B >65%. Already each samples divided into two fractions the first as a test (non progressive) fraction and the second normalize (progressive) fraction and each fraction has target gene (MT COXI) and endogenous control or reference gene (nuclear *β*-actin as normalization control) and by applying a ΔΔCq method for calculating experimental (Mt/N) ratios from means of relative Cq values obtained by qPCR analysis by using Livak method (Livak and Schmittgen ,2001) where $\Delta Cq = Cq_{(target\ gene)} - Cq_{(reference\ gene)}$ for test and normalize fraction and by find exponential expression $\Delta Cq = 2^{-\Delta Cq}$ for both and by calculation the ratio of the mtDNA/nDNA =ΔΔCq by subtracting ΔCq COXI gene from ΔCq *β*-actin therefore the amount of mtDNA determined for each patient and control groups. Infertile group that composed of 16 asthenozoospermic subjects, there was highly significant difference ($p \leq 0.05$) between the ratios of mtDNA copy number of non progressive motile sperm cells that were extracted from 40% percoll gradient which is known contained of poor sperms motility and lower relative mtDNA content corresponded to the progressive sperm-enriched fraction 80% percoll gradient on averages (160.5 ± 14.25) and (32.08 ± 6.5), respectively, as shown in (Table 3). Increasing of relative mtDNA/nDNA ratio in defective sperm fraction was ranged 74.2-271.3 copy per sperm cell (cases number 10 and 6 respectively), while the ratio of normospermic motility was ranged 1.9-92.8 copy per sperm cell as shown in (Table 4).

Results revealed there is no significant difference between progressive motile sperms of infertile subjects and progressive motile sperms of control subjects that were isolated from 80% percoll layer as well as to non progressive motile sperms of both groups that were separated from 40% percoll fraction as shown in (Table 3). The average mtDNA copy number per cell in each semen fraction 80% and 40% normospermic motile and non progressive motile sperms were recalculated taking in consideration its cell composition.

Table 3. Relative mtDNA copy number per sperm cell of mitochondrial COXI gene in progressive and non progressive motile sperms of asthenozoospermia within 0-5% group classified according to WHO criteria

Groups	Relative ΔCq		*P*-value	MtDNA copy number/ sperm cell
	Prog. [4]	Non prog. [4]		
Control	58.99 ± 22.68 [3]	119.9 ± 26.89	0.09 ns	60.90 ± 6.83
Patients	32.08 ± 6.50	160.5 ± 14.25	0.0001** [1]	128.4 ± 14.76
P-Value	0.20 ns [2]	0.16ns		0.0009**

1- ** means ± the is highly significant at ($p \leq 0.01$).
2- ns means no significant difference.
3- means ± SE.
4- Progressive prog. and non prog. mean progressive and non progressive motile sperm, respectively.

As expected, the calculated values were strongly significant difference ($p \leq 0.01$) with the mtDNA/nDNA ratio found in the whole asthenozoospermic group. Results revealed a high significant difference ($p \leq 0.01$) between defective sperm subjects and fertile samples on average (128.4 ± 14.76) and (60.90 ± 6.83), respectively, as shown in Table (3). There was clearly increasing in mtDNA content in infertile more than in control samples were 10.26 -246.97 and14.4-94.7 /copy per sperm cell respectively, as shown in Table (4). It is known that each mature mammalian sperm contains approximately 50–75 mitochondria and one copy of mtDNA (Michaels *el al.,* 1982). In another study the mtDNA copy number per spermatozoon was reported to be 75 (Hecht *et al.* 1984) or 10 to 100 (Shitara *et al.,* 1998), or 10.1 (May-Panloup *et al*., 2003) and 1,300 (Manfredi *et al.,* 1997) and 700 (Díez-Sánchez *et al*. 2003) copies for the mouse and human. The results of this study for all groups, including the control group were in line with a study carried out by Amaral *et al*. (2006) that included 93 sperm samples. It was found that the percentage of mtDNA content in patients samples was less than normospermic fraction whereas the mtDNA content was significantly higher in oligoasthenozoospermia (OAT) samples than in normal samples. In another study conducted by Díez-Sánchez *et al.* (2003), they found the values 394 for progressive spermatozoa, 477 for nonprogressive spermatozoa and 2119 for round cells, the highest ratios of copies were in the non progressive sperm, while recorded normospermic fractions decrease in the number of mtDNA copy number. May-Panloup *et al.,* (2003) found that the mtDNA ratio, expressing the average copy number per sperm was determined for 98 sperm extracts 67 and 31 (100% and 40% layers) ,respectively, obtained from the 67 ejaculates. The average ratio was 10.1, while the average mtDNA copy number per sperm was 3.8 in the 67 groups of sperm isolated from 100% density layers. The average ratio was 1.4 in the group of 32 patients with normal sperm, compared with 6.1 in the group of 35 patients showing at least one abnormal WHO criterion collected from 100% density layers i.e. their results

showed significant mtDNA amplification in sperm collected from abnormal sperm samples. These results confirm the findings of our study of the asthenospermic fractions containing higher ratios than it is among progressive motile fraction. These results are consistent with almost what we found in this study, where these results which indicated that the mtDNA/nDNA ratios of nonprogressives sperm among asthenospermia groups on average (30.5-86.53) copy/ spermatozoa moreover we found in normospermic ratios an average (13.4-53) copy/spermatozoa. This is the opposite to the results of study done by Kao *et al.* (2004) which different from what we found in the present study; Kao *et al.* (2004) noted in their research (included 86 asthenspermic patients) that the content of mtDNA was 74.1± 2.0 for sperm with normal motility scores of 51%–90%. Moreover they found extremely low content of mtDNA in 6 of 13 patients with nonprograssive. The relative contents of mtDNA in sperm of these patients were between one and eight copy per cell. An explanation for these discordant results may lie in the use of different methods to quantify the mitochondrial genome. Indeed, Southern blot may lead to cross-hybridization with the large number the Southern blot method may lead to cross-hybridization with the large number of mitochondrial pseudogenes (~300) recently found in the nuclear genome (Mourier *et al.,* 2001; Tourmen *et al.,* 2002; Woischnik and Moraes, 2002).

mtDNA, and that many sperm probably contain extremely few ratio of mtDNA. It has been shown that the paternal mtDNA, found in the early embryonic stages, is rapidly eliminated, and the researchers suggest that the lack of mtDNA content in some sperm with the best fertilizing ability may also explain why the mitochondrial genome is not paternally transmitted. The functionality of the respiratory chain must therefore be temporally maintained in mature sperm until fertilization, despite the quasi-absence of mtDNA in their mitochondria (May-Panloup, 2003). Indeed, it has been shown that mtRNA transcripts remain highly stable, and that the translation of mtRNA into subunits of the respiratory chain continues actively in the mitochondria of sperm, despite the complete absence of mtDNA replication (Rantanen and Larsson, 2000). Point mutations, deletions or haplogroups of mtDNA could be involved in male infertility. In the present study we show that highly significant mtDNA amplification was found in abnormal sperm, highlighting the multiple implications of mitochondria in male infertility. First, the mtDNA content of motile sperm was found to be up to 2 times higher in sperm samples of poor quality than in normal sperm samples. Secondly, sperm collected from the 40% layers were found to have mtDNA content up to 119-fold greater than that of sperm collected from the 80% layers. This mtDNA overreplication could have two main causes. One could be a feedback process operating to compensate low respiratory chain activity, thus

Table 4. Mitochondrial copy number of progressive and non progressive motile sperm of asthenozoospermic 0-5% group

Asthenozoospermic Subjects				Control Subjects		
Case No.	Copy number/Non progression	Copy number/ progression	Copy number/sperm cell	Copy number/Non progression	Copy number/ progression	Copy number/sperm cell
1	137.39	1.93	135.45	113.34	18.63	94.70
2	144.18	7.47	136.70	106.83	53.39	53.43
3	234.79	41.85	192.94	65.36	26.97	38.39
4	201.45	29.24	172.21	14.56	0.179	14.40
5	176.00	35.61	140.38	73.73	24.43	49.300
6	271.29	24.31	246.97	52.13	10.96	41.16
7	185.90	50.47	135.42	122.75	67.81	54.93
8	131.10	26.54	104.55	84.43	17.88	66.55
9	103.00	92.8	10.26	112.81	46.86	65.94
10	74.20	12.79	61.40	216.49	130.89	85.60
11	127.09	73.79	53.30	372.03	282.85	89.20
12	150.28	12.5382	137.74	104.06	26.94	77.11
13	118.18	23.30	94.88			
14	256.22	60.82	195.40			
15	157.53	13.88	143.64			
16	99.04	5.92	93.12			

These nuclear pseudogenes share high levels of similarity with mtDNA and might have been erroneously recognized by the wide-ranging mtDNA probes such as those used in the Southern blot technique. In contrast, PCR method in this study was demonstrated to be mtDNA specific since no false amplification of nuclear pseudogenes in cells without mtDNA was detected. Another explanation for the discrepancy of the various results reported might lie in the presence of round cells (mainly germinal cells and leukocytes) in the sperm preparations. Contamination by only a few of these cells, which have an mtDNA content exceeding that of sperm by a factor of >100, could easily lead to a serious overestimation of mtDNA content in sperm. The motile sperm extracted from normal sperm samples were found to contain mtDNA molecules ranged (13-53) among different groups. This means that the some of sperm mitochondria are almost devoid of

leading to an increase of mtDNA. Indeed, such compensatory processes of increased mitochondrial biogenesis are frequently observed in mitochondrial pathology. Another cause disturbed process could generate high levels of nonprogressive cells or a low number of spermatozoa. Semen is enriched in cells with intermediate or high mtDNA/nDNA ratio. Our results are in accord with the previously described downregulation of mtDNA copy number during rat (Rantanen *et al.* 2001) and mouse and human ((Rantanen and Larsson, 2000) spermatogenesis. The integrity of the mitochondrial genome is particularly important in sperm cells with high energy demands, such as sperm. Its DNA Polymerase Gamma (POLG) and mitochondrial transcription factor A (TFAM). TFAM activates mtDNA transcription and thus replication, and is thought to be a regulator of mtDNA copy number. Down regulation of TFAM was proposed to cause the 10-fold

reduction in mtDNA copy number during spermiogenesis. Furthermore, low levels of expression of either of these two proteins have been associated with mtDNA-depletion syndromes (Amaral *et al*., 2006). This phenomenon contrasts with and can appear paradoxical to the fact that sperm motility is a highly ATP-demanding process that clearly depends on mitochondrial oxidative phosphorylation activity. Other tissues or cell types increase their mitochondrial number and mtDNA content with higher ATP demands (Moraes *et al.* 1991, Poulton *et al.* 1995, Campos *et al.,* 1998). Closer examination of the profound effects of spermatogenesis is needed to clarify the apparent paradox. Depending on data published elsewhere (Homyk *et al.,* 1990, Auroux *et al.* 1985), there is a 40-fold reduction in cell volume (from 1500 mm^3 to 40 mm^3) during spermatogenesis in parallel with a 5- to 6-fold reduction in mtDNA copy number. As a consequence of the cellular volume reduction, a 7-fold increase in mtDNA copy number per cubic micrometer of cell is concurrent with the higher ATP requirement during spermatozoon maturation. Thus, despite the decline of mtDNA per cell, mtDNA copy number per cell volume increases as the energy requirement for the spermatozoon increases.

Conclusion

Present study showed that sperm mtDNA is highly amplified in sperm samples from asthenozoospermic patients presenting abnormal sperm characteristics. This raises the question about the risks involved in the use of a number of methods of assisted procreation, such as intracytoplasmic sperm injection (ICSI) and round spermatid injection and we suggest that mtDNA content may serve as a useful indicator of sperm quality and that mtDNA depletion may play an important role in the pathophysiology of some male infertileity.

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