

The Impact of *Toxoplasma gondii* on Mitochondrial DNA of Sub-fertile Men Sperms

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ABSTRACT

Toxoplasmosis is the most common, widespread disease in the world which is caused by *Toxoplasma gondii*. The objective of the current study is to determine the effect of the *Toxoplasma gondii* infection on male sperm, especially on the mitochondria of sperm for men who suffer infertility and the possibility of a hereditary mutation. Sixty seminal fluid and serum samples were taken from sub-fertile patients who attended Teba center for in vitro fertilization / Babylon and similarly samples were also obtained from healthy individuals as a control group, their ages ranged from 20 to 60 years old during the period from 1st may /2016 till 25th January/2017. All samples subjected to the tests included Macroscopic and microscopic examination, molecular methods was used for DNA extraction, amplification and sequencing. Statistical analysis showed non-significant differences ($P < 0.01$) among age groups and) between rural 41 (68%) and urban 19 (32%). However, sperm motility and count recorded highly significant differences ($P < 0.01$) in the mean value for healthy (74.85, 68.017) as compared with the patients (10.30, 10.217). There were also a significant differences ($P < 0.01$) in the mean concentration of Ant-*Toxoplasma* IgG antibody (1.327 μ /ml) in comparison with control group (0.564 μ /ml). A highly significant differences ($P < 0.01$) was detected when comparing the positive 55 (92%) and negative 5 (8%) samples for oligospermia and a significant difference ($P < 0.05$) for Asthenospermia 21 (35%), 39 (65%). Two mutation 2 (3%) were detected in the exchange for fifty eight 58 (97%) with a highly significant difference ($P < 0.01$). It was found that the mutation occurred in Asthenospermia of ND1 gene in the site SNP T4216C (T to C) and the site SNP C3450T (C to T) of Mitochondrial DNA. This study confirmed that a mutation in the ND1 gene that is located in the mitochondria of sperms, specifically in people who suffer from infertility, is occurred due to *Toxoplasma gondii*.

Keywords: *Toxoplasma gondii*, Molecular methods, genetic mutation.

INTRODUCTION

Toxoplasmosis is the most common, widespread disease in the world which is caused by *Toxoplasma gondii*. This parasite is transmitted by several ways, and recent studies have proven that this parasite is possible to be carcinogenic¹. Domestic and wild cats which belong to the Feline family consider to be as definitive host while, warm-blooded animals including human represent as

intermediate host. Human toxoplasmosis classified as food and water born disease, generally occurs through consuming the infective stage oocysts or tissue cysts containing the bradyzoites form. Also transmission by organ transplantation and through placenta (from infected mother to fetus) were documented as routes of infection². Some clinical studies have documented that *T. gondii* affect reproductive parameters of men , recent studies have demonstrated the transmission of the disease

through sexual contact from the husband to his wife and vice versa³. As similar studies have proven that sexual transmission has satisfactory complications including infertility⁴. Some studies have indicated that this parasite works on abnormalities in the mitochondria, of sperm, causing their death, thus infertility occurs in people with *T. gondii* infections⁵.

In the current work, the effect of toxoplasmosis on reproductive system in men with the effect of genetic mutation of sperms in the infertility incidence was studied for the first time in Iraq. The mutation velocity of mitochondrion of sub-fertile men DNA has been expected to be 10 fold more than that of nuclear DNA^{5, 6} because the presence of mutations is associated with a diversity of human being diseases^{7, 8}, reliability of mt DNA duplication, appear to lack a competent of DNA renovate system compared with the nuclear DNA repetition system⁹ and also lacks safety of histone protein. Therefore, mutations may reason from DNA damage caused by peripheral factors⁽¹⁰⁾. Abnormal sperm has a direct effect on pregnancy rates. Here's an overview of how azoospermia, oligospermia, and asthenospermia and other sperm health problems affect pregnancy. The low number of sperms is considered one of the most important reasons leading to fertility issues In couples who have difficulty getting pregnant, Guo J. *et al.*¹¹.

MATERIALS AND METHODS

Patients and control Group

Sixty samples for each seminal fluid and sera of both healthy individuals and patients of sub- fertile men were included in this study and whose ages range from 20-60 years old who attended TEBA center for children / Babylon during the period from the beginning of may / 2016 to the last of January/2017. Patients were found to be suffering from infertility either with Asthenospermia, or oligospermia.

Seminal Fluids Analysis

Sample collection

The samples were collected after a minimum of (3-5) days of sexual intercourse. Samples were kept from temperature changes until they reached the laboratory (20°C > save samples >40°C).

Serum samples

For the purpose of measuring the concentration of Anti-Toxoplasma IgG in the serum of both healthy and patients. Two to three ml was collected in sterile tubes and kept in the freeze until use.

Macroscopic examination

Liquefaction

The sample of the seminal fluid is liquidated at room temperature within half an hour, although the liquefaction usually takes about a quarter of an hour.

Volume

For the purpose of measuring the size of ejaculation samples, a cone-based cylinder was used.

Viscosity

The viscosity (sometimes referred to as "consistency") of the liquefied sample should be recognized as being different from coagulation.

pH

For the purpose of measuring pH, a drop of semen was placed on the pH paper (range: 6.1 d" pH e"10.0 or 6.4 d" pH e" 8.0).

Microscopic Examination

Preparation of semen samples

After liquefaction, one drop of 10µl of homogenized semen was placed on a warm clean microscopic glass slide with a micropipette and covered with a cover slip of 22mm X22 mm.

Sperm count

The count of sperms (how many million sperms are there in one ml of each seminal fluid samples) was estimated by calculating the mean number of spermatozoa in 10 random microscopic fields multiplied by million (10⁶).

Percentage of motility and degree of activity for sperms

Ten random fields were examined and the percentage of moving sperm was calculated, by using equation:

Motility =Number of motile sperm in I (number) of

fields multiplied by 100 divided by total number of sperm in I fields.

DNA analysis of Mitochondria (mt DNA):

Extraction and Amplification of mitochondrial DNA

Mitochondrial isolation kit (Biovision) was used for mt DNA extraction . Agarose gel electrophoresis (1.2%) was used for visualization of extracted mt DNA as gel loading buffer was prepared by mixing three μ l of the extracted DNA with 3 μ l of Orange G (30% glycerol and 35% Orange G). Electrophoresis was done using 1x TBE buffer at 5 V/cm for half an hour. Gel were stained with ethidium bromide (half a gram/ml) and then visualized by using an Ultra- Violet trans illuminator (300 nm).

The UV spectrophotometer device was used to determine the concentration and purity of the extracted DNA. The concentration was calculated using absorbance at 260 nm reading (one optical density unit) which corresponds to 50 μ g/ml DNA.

PCR reaction and Application

Two sets of primers were designed, for ND1 mitochondrial genes amplification. The reactions of PCR were optimized for each primer pair with different thermal grades used for annealing process. Approximately 160 ng of total sperm DNA was used in 50 μ l of reaction mixture. The PCR reaction mixture contained the following:

1.5 μ Mg²⁺

200 μ M of deoxynucleotide triphosphate (dATP, dCTP, dTTP, dGTP)

1 \times Taq buffer, 0.6 μ M of each primer and

1 unit of Taq DNA polymerase (Roche).

During PCR cycle, temperature 94C^o was used for two minutes in the initial denaturation process, followed by 35 cycles of denaturing within the same temperature but for 30 second , annealing was done at 58C^o for 45 second and extension of primers was carried out at 72 C^o for 2 min. while the final extension required 5 min. at 72 C^o .The reaction products were saved at 4 C^o.

The primers for the ND1 gene are

F 52 -ACATACCCATGGCCAACCTC-32 and R 52 -AATGATGGCTAGGGTGA CTT-32 .

52 -ATGTTTGAGACCTTCAACAC-32 and

5-CATCTCTTGCACGAAGTCGA-32 respectively.

DNA Sequencing

Automated mt DNA sequencing of the ND1 gene was performed for different fertility groups using the Bio- Applied System, dye TM termination V 3.1 cycle sequencing kit.

Evaluation of DNA Sequencing

All the data obtained from automated sequence was edited with a computer based program sequencer TM. To determine the nature of mutations, compare with the reference sequence (NCBI database, accession number: NC_00187) has been adopted.

Analysis for Silent Mutations

The synonymous mutations were analyzed for codon usage. Data obtained from this study and data from Bail and Bebok 2016⁽¹²⁾ were analyzed for identification of amino acid changes by the computer program MEGA version 21¹³.

Analysis for Non-Silent Mutations

Hydrophobicity and polarity were used in protein analysis for the purpose of determining the effect of amino acid changes on protein properties , this was done with computer based program (<http://www.roselab.jhu.edu/~raj/MISC/hphobh.html>) and ProtScal (http://download.invitrogen.com/evergreen/support_files/a-protscale-expasy.htm)

Statistical analysis

The program SPSS version 16 and Microsoft Office Excel 2007 were used for data analysis. Numeric data were expressed as mean \pm standard error of mean (SEM). Unpaired t-test and chi squared was used to compare numeric data for healthy control and patients groups while Chi-square was used to assess the differences between the proportions. P-value <0.05 was considered significant.

RESULTS

The distribution of Toxoplasma infection among studied groups according to the age and the residency

In table one , the demographical picture for the age group showed that from the sixty total

samples 24 of healthy men (40%) and 21 of patients (35%) were aged between 20-40 years old while, 36 (60%) and 39(65%) were aged between 41-60 years old for healthy and patients respectively. There were no significant differences ($P<0.05$) when comparing the two groups. According to residency, no significant differences ($P<0.05$) was recorded when compared between rural 39(65%), 41(68.3%) and urban areas 21(35%),19(32.7%), for healthy and patients respectively.

According to the results in table two, highly significant differences ($P<0.01$) were observed in both sperm motility and count when comparing two groups. The mean of sperm motility and sperm count was 74.85, 10.30 and 68.017, 10.217 for healthy and patients respectively.

Demographical Picture of Anti- Toxoplasma antibodies IgG, among the studied Groups

Results in table three revealed a highly significant difference ($P<0.01$) in the concentration

of IgG antibodies of patients group in contrast with healthy group. The mean concentration of IgG was 0.564 μ /ml for healthy and 1.327 μ /ml for patients.

Positive and negative cases of Oligospermia, Asthenospermia, and mutation in seminal fluids among sub-fertile patients

Table four elucidate that for both of oligospermia sperm and mutation in ND1 gene of mitochondrial DNA, there was a highly significant differences ($P<0.01$) between positive 55(92%), 2(35%) and negative 5(8%), 58(97%) sub-fertile patients respectively. While, the Asthenospermia showed significant difference ($P<0.05$) between positive and negative 21(35%) , 39(65%) respectively.

Amplification of mt DNA

PCR reactions were performed to amplify the ND1 genes from the extracted DNA samples. Among them, twenty-one sample showed the appropriate product size of the DNA (1183 bp) with ND1 were digested with restricted enzymes, EcoRI

Table 1: Distribution of Toxoplasma infection among studied groups according to the age and the residency:

Parameters	Studied groups			Chi-Square Test(P-value)
	Healthy (N= 60)	Patients (N= 60)	Total (N=120)	
Age groups / Year	20 – 40	N	24	X ² value=0.32 P=0.572
	%	40%	35%	
Residency	41 – 60	N	36	X ² value=0.15 P = 0.699
	%	60%	65%	
Residency	Rural	N	39	X ² value=0.15 P = 0.699
	%	65%	68.3%	
Residency	Urban	N	21	X ² value=0.15 P = 0.699
	%	35%	31.7%	

Table 2: Sperm motility and count in healthy and patients samples

Parameters	Test groups	No.	Mean	TD	S.E	t-test (P-value)
Sperm motility%	healthy	60	74.85	11.663	1.506	P <0.01
	Patients	60	10.30	7.021	0.906	
	Total	120				
Sperm count million/ml	healthy	60	68.017	11.1985	1.4457	P <0.01
	Patients	60	10.217	5.6869	0.7342	
	Total	120				

and HindIII. Figure (1) shows ND1 PCR products for 5 samples.

DNA Sequence analysis: (ND1 gene)

After purification of PCR products, automated DNA sequencing was performed using the Applied Bio- System Big Dye TM termination V 3.1 cycle sequencing kit. Twenty one of sub-fertility samples were sequenced for the ND1 gene⁽¹⁴⁾. These sequences were compared with the reference value obtained from the National center for biotechnology information (NCBI) database. A computer software program Sequencer TM was used to edit the gene sequences and for determination the nature of mutations. Two mutations in different nucleotide position in ND1 region were found in different groups of the samples. The base substitutions were located at nucleotides (nts) 3450, 4216 (Table 4).

7-Single nucleotide polymorphisms (SNP) T4216C

The evolution of T to C was notorious at nt 4216 in the ND1 gene. This SNP was found in an Asthenospermia of man (sample code, 010480). This SNP is an equivalent changeover in the third

position of a tyrosine codon, altering it from TAC to TAT at nt 3480 (Figure 2, Table 4).

Single nucleotide polymorphisms (SNP) C3450T

The evolution of C to T transition was identified at nt 3450 in ND1 region. This transition was observed in an asthenozoospermic (code 6445) sample. This SNP, a synonymous substitution occurred in the third position of the proline codon, changing the codon CCC to CCT (Table 4).

Analysis of Codon Usage in Synonymous Mutants in Mitochondrial DNA

The frequency of codon usage for each mitochondrial gene was determined using the computer program MEGA version 6.06,⁽¹³⁾. The amino acid and their frequency change rate were determined. Analyses of codon usage in synonymous mutants between fertile and Asthenospermia are tabulated in Table 4.

Statistical Analysis for Gene Sequencing

A statistical analysis was performed on individual SNPs that were identified in at least 2 samples of the total fertile and sub-fertile population.

Table 3: The concentration of IgG antibody in serum among studied groups

Parameters	Test groups	No.	Mean μ/ml	S.D	S.E	P
Serum Anti-Toxoplasmosis IgG Ab.	healthy	60	0.564	0.201	0.026	<0.01
	Patients	60	1.327	0.584	0.076	
	Total	120				

Table 4: The positive and negative numbers of abnormal cases in seminal fluid of sub-fertile patients

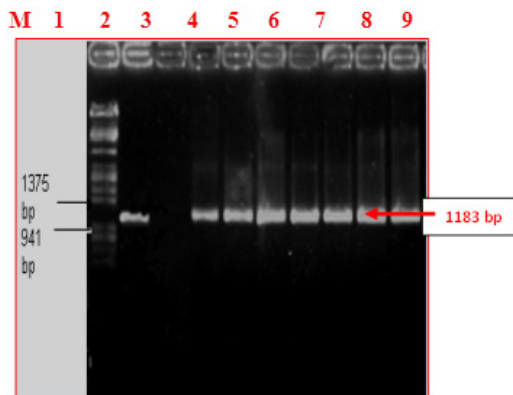
Parameters for Patients	No.	Percentage %	P
Oligospermia	+ve	55	< 0.001
	-ve	5	
	Total	60	
Asthenospermia	+ve	21	0.027
	-ve	39	
	Total	60	
Mutation in ND1 gene of mt DNA	+ve	2	<0.001
	-ve	58	
	Total	60	

The Z value (binomial test) was determined for testing of significant differences between the mutants/polymorphisms in the sets of fertile and sub-fertile men. The Z value was calculated from $Z = |P1 - P2| / \sqrt{P(1-P)/n}$ ¹⁵.

DISCUSSION

This study was conducted for the first time in Iraq, to study the possibility of the effect of toxoplasmosis on infertility of the men. The results revealed a non-significant differences between the age groups (42.55±6.753) in comparison with control

group (40.37±7.863) among the toxoplasmosis patients, this is in line with the finding of many studies such as (Mohammed 2016)⁽¹⁾ and (Singh, 2016)⁽²⁾. Non-significant difference was recorded between rural 41(68%) and urban 19(32%) according to residency this result is in agreement with (Singh, 2016)⁽²⁾. The result also demonstrated that there were highly significant differences ($P < 0.01$) regarding to the elevation of the concentration of Ant-*Toxoplasma* IgG antibody (1.327±0.564) in comparison with control group (0.564±0.201) among studied groups. This is consistent with Thamm *et al.*, 2016,⁽¹⁶⁾ who identified that by using application of multivariate regression has increased of seroprevalence from 20% (95%-CI:17–23%) in the 18–29 age group to 77% (95%-CI:73–81%) in the 70–79 age group⁽¹⁶⁾, these results also agreed with Ollivier *et al.*, 2016⁽¹⁷⁾, and Islamorada, 2013, whose studies showed that chronic toxoplasmosis affected on reproductive parameters in men,¹⁸. Nevertheless, there was a significant increase between the census of sperm count and the motility of sperm in similitude with control groups (10.217± 5.6869), (10.30±7.021), (68.017±11.1985). (74.85±11.663), respectively between patients with toxoplasmosis infection. These results are congruence with Dalimi, 2013⁶, who demonstrated that toxoplasmosis was highly impact on the production of sperm and its vitality and effectiveness,⁶. Shiadeh, *et al.*, 2016, proved in a study that *Toxoplasma gondii* could cause endometritis, impaired folliculogenesis, ovarian and uterine atrophy, adrenal hypertrophy, vasculitis, and cessation of estrus cycling in female and in male cause decrease in semen quality, concentration, and motility¹⁹. While Ab-dulla, *et al.*, reported that during infection, each of the parasite and the host used various mechanisms to enhance their own reproductive success, along with an ever-growing



Lane M: Lambda DNA marker digested with restricted enzymes, *EcoRI* and *HindIII*.

lane 1: Positive control (sample containing extracted DNA of correct PCR product size for ND1).

Lane 2: negative control (sample without extracted DNA).

Lane 3 4,5: 6,7,8 and 9: asthenozoospermic

Electrophoresis was done on an agarose gel (1.2%) using 1 × TBE buffer and visualized by using an ultra-violet (UV) transilluminator (300 nm).

Fig. 1: PCR products of ND1

Table 5: DNA sequence analysis and the codon usage of synonymous mutations of semen samples in mitochondrial DNA

Cases	Gene	Mutation	Codon change	Amino Acid Change	Codon Frequency Change
Asthenospermia	ND1	T4216C*	TAT-TAC	Silent	1.6-1.63
Asthenospermia	ND1	C3450T*	CCC-CCT	Silent	1.04- .087

* indicates the mutations obtained from different groups of semen samples

number of couples suffering from idiopathic infertility. Although there is a lack of research on how *T. gondii* can alter reproductive parameters, one of the common methods for determination of fertility status in males is seminal fluid analysis ⁽⁴⁾. In the present work, the results showed that a significant differences for each of oligospermia, 55 (92%) were positive and 5 (8%) were negative that a highly significant difference ($P < 0.01$), while the Asthenospermia 21(35%) and 39(65%), with a significant difference ($P < 0.05$) and mutation were 2(35%) were positive and 58(97%) highly significant ($P < 0.01$). This study was performed for first time in Iraq, and has proven that there was a genetic mutation in the base substitutions and that there was a change in SNP T4216C position, T to C transition was identified at nt 3396 in the ND1 gene. This SNP was found in an Asthenospermia man (sample code 010573), and replace synonym in the third position of a tyrosine codon, changing it from TAT to TAC, Another SNP was found at C3450T position, C to T transition was identified at nt 3450 in ND1 region. This transition was observed in an asthenozoospermic (code 6445) sample and replace synonym in the third position of the proline codon, changing the codon from CCC to CCT, these results were obtained

using the analysis of codon usage in synonymous mutants in mitochondrial mt DNA¹³. Other studies reported obtaining genetic mutation that assigned the current study. Whereas these results were in a harmony with Hoang, *et al.*,²⁰ they revealed that mutation from either exogenous or endogenous sources, like DNA replication errors or environmental insults like smoking or sunlight and they developed the bottleneck sequencing system (BotSeqS) as a simple genome-wide sequencing-based method that accurately quantitates nuclear and mitochondrial mutational load in normal human tissues also, they demonstrated that mutation prevalence and spectrum vary depending on age, tissue type, DNA repair capacity, and carcinogen exposure. These results suggested a varied landscape of rare mutations within the human body that has yet to be explored¹⁹. Also there is convergence with Chen *et al.*, (2016)²¹, who found that a comprehensive screen of 874 genes in 589,306 genomes led to the identification of 13 adults harboring mutations for 8 severe Mendelian conditions, with no clinical manifestation of the indicated disease. Our findings demonstrate the promise of broadening genetic studies to systematically search for well individuals who are buffering the effects of rare, highly penetrant,

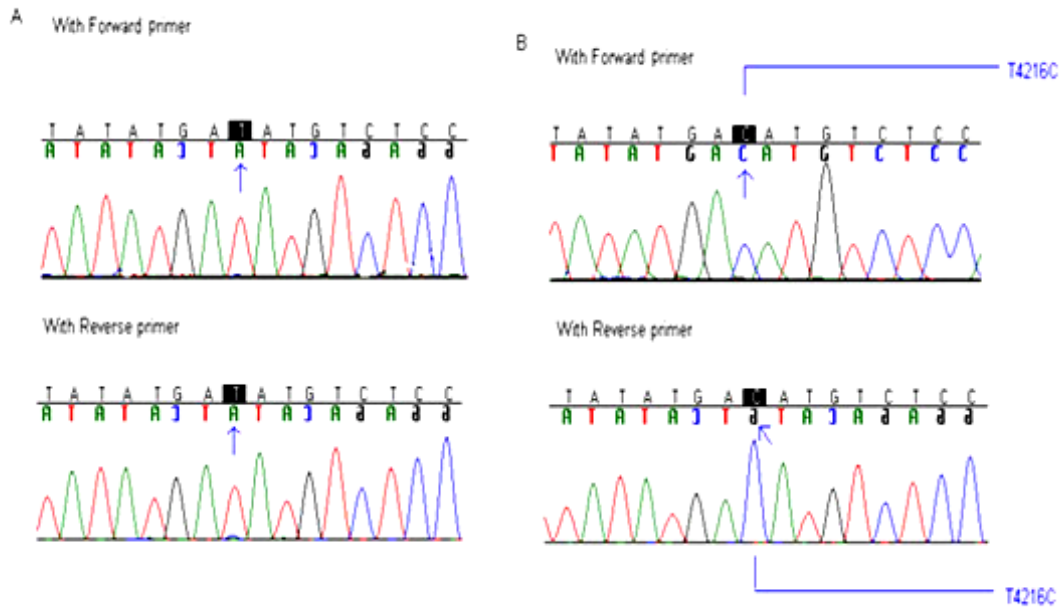


Fig. 2: Single nucleotide polymorphisms T4216C

deleterious mutations²¹. Therefore, this study was conducted to determine a genetic mutation in the ND1 gene that is located in the mitochondria of

sperms, specifically in people who suffer from infertility, and based on these previous studies. This study demonstrated that this mutation occurred due to *Toxoplasma gondii* infections.

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