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Original paper

The role of chronic infection with toxoplasmosis in mitochondrial DNA 4,977 bp common deletion in sperm of men infertility

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ABSTRACT. *Toxoplasma* has the ability to infect a wide range of living organisms, including humans, and the infection found all over the world. The current study included 200 blood and semen samples collected from married men from which 100 samples of infertile men and 100 non-infertile men as control. The infertile men were divided into two groups: group A, which includes 35 (35%) infertile patients have IgG antibody sero-positive with toxoplasmosis, and group B, which includes 65 (65%) infertile people without toxoplasmosis were detected by rapid test for Toxo IgG/IgM antibody and then ensure by enzyme linked sorbent immunoassay (ELISA). The aging of men involved in the study extended between 20–57 years. The study revealed group A *Toxoplasma* IgG positive have mean concentration 65.10±13.84, was measured by ELISA in the serum of patients. Studies have shown mean age of men in group A and group B patients were 32.77±7.83 and 30.31±6.14, respectively. The age from 20–29 years as well as from 30 to 39 years high number of infertility in both A and B group patients. There is no significant distribution between group A and group B patients according to age of men infertility. The common deletion 4,977 bp mutation in mitochondrial sperm DNA(mt-DNA) was detected using the Gap-polymerase chain reaction (PCR) technique. Although the results showed that group A have high rate of common deletion mutation compare to group B but non-significant, while there is no deletion detection in the control group. The current results showed that reducing the mean total number of sperms in group A patient common deletion high significant compared to group B. The mean percentage of slow movement high significant than group B patient but non-significant mean percentage of death sperm although have high mean than to group B patient and abnormal sperm shape percentage have significant is higher in group A patient (common deletion) compared to group B.

Keywords: toxoplasmosis, mitochondrial DNA 4,977 bp, men infertility

Introduction

Toxoplasma gondii was first found by Nicolle and Manceaux in hamster-like rodent tissues called common gundi (*Ctenodactylus gundi*) [1]. A protozoan parasite that pass on a disease to human and almost other warm blood animals and compromised one of most common pathogens in eukaryotes [2]. The infection is chronically of *T. gondii* in the world population of human about 30–50% [3,4]. *T. gondii* have significant in human health and veterinary and the study of unicellular microorganisms at the level of biological and molecular [5].

Although there were much data on sterility in other countries, little exist on infertility in Iraq [6]. It is a heavy problem on uncountable families, with significant implications for both individual and public health [3,7].

Some recent studies have showed the impact of toxoplasmosis on reproductive system in men with the consequence of genetic mutation (ND1 gene) of sperms in the sterility men existence [8]. The mutation rapidity of mitochondrion of sub-fertile men DNA(mt-DNA) has been probable to be 10 fold more than that of nuclear DNA [9].

The identification and genetically characters of *T. gondii* infection is fundamental investigation, inhibition and manager of *T. gondii*. Old-style methodologies to the finding of *T. gondii* involve

etiologically, immunologically and image methods while toxoplasmosis discovering can enhance through the amplification parasitic DNA molecules based on molecular characterization. Amongst this polymerase chain reaction (PCR) procedure have been suitable for the genetically characterization of parasitic infection [10].

Gap-PCR or long range PCR amplifies the lost DNA sequence using the primers nearby the deleted region, Gap-PCR are used to detection of mutation by deletion alleles which may occur in sequence of DNA molecule, this deletion must be known and detected by primers designed specifically to flanked these deletion sequences, the products of Gap-PCR does not generated except if the sequences that flanked deletion are joining together, that can be obtain when diagnosis the PCR products using gel electrophoresis [11].

Men infertility is usually related to abnormalities of sperm comprising (asthenozoospermia), the mutations in mitochondrial DNA (mt-DNA) are greatly related with asthenozoospermia according the molecular diagnosis, common deletion (4,977-bp) in mtDNA of human one of the important mutations influence of sperm progression that result in lost approximately (33%) from the genome of mitochondrial [12]. Numerous mutations cause in mt-DNA that can related and resultant in many diseases comprising neurologically illnesses also with men sterility [13]. Numerous environmental elements have been linked with asthenozoospermic sterility like sperm exposed for long-time to infection [14]. Since the histones lack in mitochondrial genes that might cause various kinds of mutations comprising the common deletion in the mt-DNA and subsequently result to men sterility [15,16].

Current study the first training that displayed the closely relation between chronic toxoplasmosis could distress reproductive parameters and common deletion (4,977 base pair) in sperm mitochondrial DNA (mt-DNA) of infertile men. The of current study to prove relationship between men infertility chronically infected with toxoplasmosis and mt-DNA common deletion (4,977 bp) in sperm.

Materials and Methods

Blood sample collection

Five milliliters blood samples are collected by puncture the venous using sterile syringe under aseptic conditions to obtain serum sample kept in the freezing using for detection of human immunodeficiency virus antibody HIV (type 1 and 2) because consider one of topic cause male infertility, IgM and IgG *Toxoplasma gondii* in men kit was used in this study for quantitative and qualitative detection of *Toxoplasma* IgM and IgG levels in human serum samples done according to company instruction (Foresight. USA).

Semen sample

Seminal fluid samples collected in sterile container by ejaculated after (3–4) days of sexual intercourse using for general seminal analysis according WHO (2010) and for rapid test qualitatively detects *Chlamydia trachomatis* antigen, *Neisseria gonorrhea* antigen because consider one of topic cause male infertility and then stored at –20°C to used it in mt-DNA extraction for Gap-PCR technique.

Results

The infertile men and healthy control distribution according to toxoplasmosis seropositive IgM and IgG by rapid test and ELISA

The distribution of patients with infertility and healthy control in relation to toxoplasmosis seropositive IgM and IgG shown in table 1. The present findings show non-significantly differences in men infertility distribution and controls according to seropositive IgM rapid test and ELISA technique separately (P=0.51) and (P=0.081). the findings showed a highly significant differences according to toxoplasmosis seropositive IgG rapid test and ELISA technique, were all group A patients are seropositive IgG rapid test and ELISA technique respectively, (P<0.001). Also high mean levels of serum IgG in group A patients detected by ELISA technique, 65.10±13.84 IU/ml is the highly significant difference (P<0.001).

The results showed the group A patients with positive common deletion accounted for 11 (31.4%), whereas, group B patients with positive common deletion accounted for 17 (26.2%), although, the rate of positive common deletion was higher in group A patient than in group B patients, but the differences were non-significant manner (P1=0.575). But both patients (A and B) highly significantly (P2<0.001) higher than that of healthy controls (Tab. 2).

The present results showed the mean of the total sperm count of group A common deletion patient

Table 1. Distribution of infertile and fertile men (as control) according to *Toxoplasma gondii* seropositive IgG and IgM by rapid immunoassay test and ELISA technique

Characteristic	Infertile patients n=100	Control n=100	OR	*P*
		Rapid test		
Seropositive IgM				
Positive, n (%)	2 (2.0%)	0 (0%)	2.020	0.155
Negative, n (%)	98 (98.0%)	100 (100%)		NS
Seropositive IgG				
Positive, n (%)	35 (35.0%)	0 (0%)	42.42	<0.001
Negative, n (%)	65 (65.0%)	100 (100.0%)		HS
ELISA technique				
Seropositive IgM				
Positive, n (%)	0 (0%)	0 (0%)		
Negative, n (%)	100 (100.0%)	100 (100%)		
Seropositive IgG				
Positive, n (%)	35 (35.0%)	0 (0%)	42.42	<0.001
Negative, n (%)	65 (65.0%)	100 (100%)		HS
Mean of positive IgG				
	65.10±13.84	0		<0.001 HS

Table 2. Frequency distribution of group A and group B patients compared to healthy control according to common deletion (4,977 base pair) in sperm mitochondrial DNA

Variables	Group A n=3	Group B n=65	Healthy control n=100	*P*-value
Common deletion				
Yes, n (%)	11 (31.4%)	17 (26.2%)	0 (0%)	*P*1=0.575 NS
No, n (%)	24 (68.6%)	48 (73.8%)	100 (100%)	*P*2<0.001 HS

11.12±8.33 high significantly ($P \leq 0.001$) higher than group B common deletion patients 23.29±17.72. The present findings show the mean of sluggish motile sperms of group A common deletion patients high significantly higher than the sluggish motile sperms of group B common deletion patients ($P \leq 0.001$), 53.30±21.74 vs 25.23±14.64, respectively. Although the findings indicate the mean number of dead sperm of group A common deletion patient higher than the mean number of dead sperm of group B common deletion patients 54.05±20.08 vs 47.23±22.54, but the differences non-significantly (P=0.254). Also, the abnormal sperm of group A common deletion patient have mean 66.27±15.42 significantly (P=0.012) higher than the mean of abnormal sperm of group B common deletion patients 49.94±17.17 (Tab. 3).

Discussion

The results in table 1 showed non-significant differences in occurrence distribution of infertile men and controls according to seropositive IgM rapid test and ELISA technique, respectively (P=0.155) and agree with [17–21]. This may be *Toxoplasma gondii* at the beginning of the injury, the level of IgM rises, but for a short period after that it begins to decline until it completely disappears. This decrease may lead to the inability to detect it in many infected people, this agree with

Table 3. Comparison between group A (common deletion) and group B patients (common deletion) according to seminal fluid parameters

	Common deletion comparison		
	Group A n=11	Group B n=17	*P*-value
Total sperm count (*106)			
Mean±SD	11.12±8.33	23.29±17.72	0.001 HS
Range	0.00–25.00	0.00–65.00	
SE	2.51	4.17	
Motility (sluggish %)			
Mean±SD	53.30±21.74	25.23±14.64	0.001 HS
Range	17.40–100.00	0.00–52.00	
SE	6.55	3.452	
Dead sperm (%)			
Mean±SD	54.05±20.08	47.23±22.54	0.254 NS
Range	30.00–100.10	10.00–82.00	
SE	6.05	5.31	
Abnormal (%)			
Mean±SD	66.27±15.42	49.94±17.17	0.012 S
Range	50.00–90.00	10.00–100.00	
SE	4.64	4.04	

[22]. Although there is a positive IgM and it may indicate where to indicate a recent infection in people who have a chronic infection, but these results in some cases may give a false positive result, this agree with [23]. further more acute infection with *Toxoplasma* leads to simple to moderate flu-like symptoms that many infected people can overcome, especially those with strong immunity, but the parasites continue to grow slowly inside the affected tissues and enter their latent phase [24]. But the result showed highly significant differences according to seropositive IgG rapid test and ELISA technique, were all group (A) patients are positive for seropositive IgG rapid test and ELISA technique respectively, (P<0.001). The greatest communal form of *T. gondii* human's infection is asymptomatic (latent) however in some disorders like immunity compromised patients the infection may cause severe disease this agree with [19,25–27]. On the other hand, the difference highly significantly (P<0.001) in the concentration mean of serum IgG toxoplasmosis antibody in group A patients were high using ELISA technique, 65.10±13.84 UI/ml, high level of IgG-*Toxoplasma* may remain for a long time. This high titer may be indicating that the primary infection is a high infection, or that the parasite has reactivated after the infection was latent, or because the continuous response by the immune system is a result of chronic infection and *T. gondii* bradyzoite infection persist for the lifetime in the host [28]. Furthermore, the high level of IgG is related to many factors, including warm weather, high humidity, duration and severity of the infection, as well as the presence of cats, domestic animals and strays, all of which may lead to an increase the period of exposure to infection that consequence increasing in the level of IgG in the sera of the study population this agree with [29].

Distribution of group A patient and group B patients according to common deletion (*4,977 base pair*) *in sperm mitochondrial DNA in compared to healthy control.*

This is the first time study to link between infertility men infected with toxoplasmosis and a common deletion 4,977 bp mutation in their sperm mitochondrial DNA(mt-DNA) (Fig. 1). Mohammed

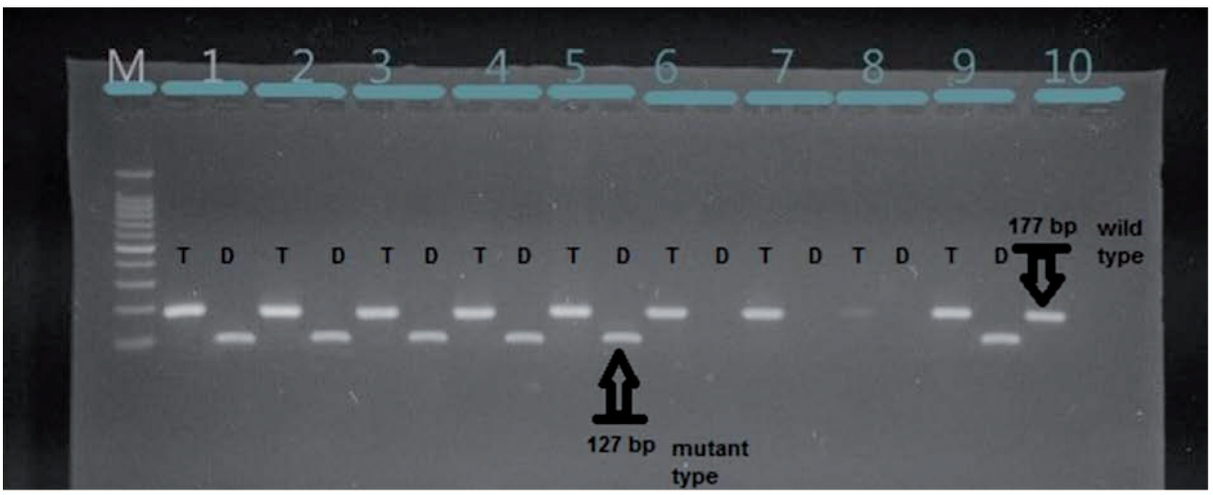

Figure 1. Agarose gel electrophoresis image that showed the Gap-PCR product analysis of mt-DNA common deletion 4,977 bp positive *Toxoplasma gondii* of patient's sperm samples
M: marker (1500–100bp); lanes (T) wild type at (177 bp) PCR product; lanes (D) deletion (mutant) type at (127 bp) PCR product; the positive results of mutant type in lane 1, 2, 3, 4, 5 and 9

et al. [8] who found that infertile men chronically infected with *Toxoplasma gondii* had a mutation in the ND1 gene, which is one of the 13 genes that encoded in the mitochondrial DNA it is an essential component of the complex respiratory chain. Toxoplasmosis infection leads to great and important damage to the DNA of the sperm in the rats [30]. The nucleic acids of *Toxoplasma* are found in the sperm, testes and epididymis of many animals [31–35] and can alterations profiles of small RNA in sperm [36]. The occurrence of the common deletion of mitochondrial DNA in sperm remains uncertain [9]. Toxoplasmosis latent infection that affects the male reproductive system is one of the most important causes that prevalence and lead to men infertility [37] as this infection may lead to an effect in weakening the sperm motility or may lead to a deterioration in the male gonads [38], *Toxoplasma* has the ability to reduce the fertility rate in male mice infected in the laboratory [39], because it has the ability to enter and persist in the semen of many animals and even humans [40] also because it has the ability to cause histopathological changes in many tissues of the male reproductive system such as the epididymis, testicles and ability to infect and present in the testicle macrophage cells, Sertoli cells and the seminiferous tubes [41] so such chronically toxoplasmosis infection caused persist activation of macrophage and leucocyte which is the another main source in the production of ROS [42], Infection with *T. gondii* may have a role in the function of chromatin abnormalities caused by increased oxidative stress [43,44]. Therefore, the direct side effects resulting from the harmful effect of oxidative stress have an effect on the reproductive system in rat male infected with *T. gondii* [45]. The increased oxidative stress in testes caused by the immune system against infection with toxoplasmosis induces the parasite to change its phase to the chronic phase [46]. The excess production of ROS from the mitochondria of the sperm is highly correlated with the defect in semen, especially with regard to the movement of sperm, so this increase in the production of endogenous ROS is one of the original sources in abnormal sperm [47]. Increasing the production of ROS may lead to the progression of the inflammatory response in the affected organs and cell death, as well as can lead to the death of sperm due to damage that may occur in the mitochondrial DNA [48]. An excess of ROS begins the process of lipid peroxidation and DNA damage, and as a result, it will lead to carcinogenesis, cell death and mutations. Therefore, the increase in phosphorylated oxidation in the sperm leads to damage to the sperm membrane, which in turn works to affect the shape, movement, and fusion of sperm with oocysts [49]. The functional abnormalities occur in sperm cells by the stress of oxidation is the major participate in men idiopathic infertility [44]. There are two main reasons for the occurrence of infertility as a result of the increase in ROS which leads to direct DNA damage to the sperm, or through damage that occurs in the plasma membrane of the sperm, which leads to a decrease in its movement and thus reduces its ability to fertilize oocysts [50]. Oxidative stress has the

ability to damage all molecules in the cell, such as lipids, nucleic acids, and even proteins [51]. Also mt-DNA is more sensitive to damage because it represents the prime target of the phosphorylated oxidation process, and this type of oxidative damage may lead to a process of deletion in mitochondria resulting from the separation of the two DNA strand, so common deletion (large scale deletion) is one of the most important changes in Mitochondrial DNA, which leads to many diseases in humans [52]. Sperm that have damage or defect in the mitochondria leads to a decrease in energy production, as well as an increase in ROS and free radical, which in turn leads to a defect in the production of energy needed for the movement of sperm, which leads to infertility in men [53]. An increase in oxidative stress renders the respiratory chain homeostasis impaired through damage to mitochondrial DNA that results in an adverse effect on membrane penetrability, calcium homeostasis and a defect in the mitochondrial protection system [54]. The deletion caused by the mitochondrial genome in sperm is a partial or complete deletion of the genetic structure in the genes of the respiratory chain, which are ND3, ND4, ND4L, COIII, ATPase 6, ATPase 8 and five of tRNA [55]. This type of mutation is the most common in mitochondrial DNA, in which one-third of the length of the mitochondrial DNA is removed or lost and thus fused ND5 with ATPase 8 which leads to a decrease in ATP production and thus reduces the production of energy needed for movement [16].

In other hand, *Toxoplasma* infection has a complex relationship with the host mitochondria and this effect is characterized by the modulations of the host mitochondrial which includes the production of superoxide and the expression of phosphorylated oxidation proteins, immune response as well as a change in the form of the mitochondria [56].

Although the result revealed non-significantly but the rate is higher in group A patients (31.4%) than that of group B patients (26.2%) this may be belong to the small size of sample. With regard to patients of the group B the common deletion 4,799 bp may be present in several other diseases, such as cancer of the skin, neck, prostate, stomach, thyroid, mouth, head, esophagus as well as in the breast and colorectal [58]. In addition, 40% of patients with mitochondrial myopathological diseases have a deletion in 4,977 bp, furthermore, the 4,977 bp deletion, which is associated with many mutations in tissues and organs in the human body, including the brain, skeletal muscles, the heart and in skin photo aging, therefore is used as a biomarker [59]. Also during the development process, the DNA and chromatin of the sperm may be damaged as a result of various internal and external conditions [60]. DNA production may be inhibited by mutations that result from direct DNA damage or during cellular death [61].

Comparison between group A patients common deletion and group B patients common deletion according to seminal fluid parameters

The finding agree with Wang [62] who suppose *Toxoplasma gondii* have ability to direct infected of human reproductive system because have ability to cross the testes blood barrier and agree with [63] who concluded in their study that chronic infection with *Toxoplasma* is associated and has a significant effect on many diseases that affect humans and other laboratory animals reproductive organ with impact on the morphology, movement and count of sperm [37]. Infection with *Toxoplasma* leads to DNA damage in the semen, and thus it will decrease the semen parameters including number, morphology, movement and viability of sperms which leads to a decrease in the fertility rate in infected mice [30]. Thus the parasitic infection can lead to persistent inflammation and such inflammation in male reproductive system involving testes and epididymis have important in reduce the sperm count, motility and morphology that can lead to male infertility [64]. The remarkable epigenetic evolution of the parasite its ability to relocate the host cell's mitochondria around the parasitophorous vesicle formed by *Toxoplasma* with help of virulence factors effector proteins secreted by *Toxoplasma* [65]. Thus the binding of parasitophorous vesicles to host mitochondria is rapid and have an important role in modulating the immunity response to *T. gondii*, here the effect of gene expression of genes involved in the oxidative phosphorylation process that causes a defect in the host mitochondria of infected cells appears [56]. The first stage of the fertilization process sperm need a large amount of ATP in order to move the flagellum, which contains about 70 to 80 mitochondria in the mid zone in humans and other mammals sperm [66]. Thus bioenergetics that mitochondria confer on sperm is definitive for sperm motility, so any defect in qualitative as well as quantitative characteristics in mitochondrial

DNA will adversely affect all cellular functions of sperm [67]. Also the process of generating sperm in humans is affected by any defect that may occur in the activity of respiratory oxidation chain in mitochondria, especially spermogenic as a result of the accumulation of mutations in the mitochondrial DNA of sperm in the testes, which leads to a decrease in energy production by the mitochondria, which may induce the stop of division in the sperm-generating cells [15]. Chronic parasitic infection and the formation of cysts within the tissues generate antioxidants during infection thus generate oxidative stress in these tissues, including the testicle caused accumulation and increase of oxidative stress leads to damage the sperm chromatin [68] that consequence may be affect to sperm count. Therefore, sperms that have damaged DNA or defects in chromatin fail to form sperm capable of being fertilized [69,70]. Since any mutation including common deletion 4,977 bp that occurs in the mitochondria DNA disturbs the production of ATP, which in turn leads to a defect in the production of sperm and the movement of the flagella and the process of the mitochondria depleting the sperm mainly affects the decrease in sperm motility and its fertility [15]. Of all the mutations that occur in mitochondria, common deletion mutations are the most incidence and abundant [71] and such deletion important related with abnormal sperm and men infertility in wide range of population in the world [72,73]. the main reason for occurrence of 4,977 bp large scale deletion is endogenous source accomplished by ability of chronic *T. gondii* to induced over production of ROS and to change cellular metabolism and molecular level of the infected host [74].

In conclusion, the current study concluded that infection with *Toxoplasma gondii* can caused common deletion 4,977 bp in mt-DNA of sperm in infertile men and such mutation can effect on sperm parameter in infected men which leads to male infertility.

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