The Role of *Protamine 1 Gene* Polymorphism and Anti FSH Antibody in Infertile Couples

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الخلاصة:
الهدف من هذه الدراسة هو تقدير دور جين1 Protamine المتعدد الاشكال في عقم الرجال وكذلك تقدير الأجسام المضادة للهرمون المحفز للجر يبات في دم النساء؟
اجريت هذه الدراسة على (72) زوج (72من الذكور، 72 من الاناث) يعانون من العقم وبمدى عمر بين(18-52) سنه ,شوهدت الحالات في مستشفى الديوانية التعليمي بمدينة الديوانية في الفترة من كانون الاول 2012 وحتى ايار 2013 ، ومجموعة اخرى (20) زوج اصحاء (20 ذكور , 20 اناث) كمجموعة سيطرة. تم جمع عينات الدم من كلتا المجموعتين وتم استخلاص الحامض النووي الجيني (DNA) من كريات الدم البيضاء في الدم المحيطي, ولمزيد من الدراسة الجزيئية للكشف عن أي ارتباط بين الطفرات الوراثية في جين (G197T) وعلاقة ذلك بعقم الرجال تم استخدام تقنية انزيم البلمرة المتسلسل (PCR-RFLP) لهذا الغرض وهضم الحامض النووي المستخلص بواسطة انزيم (*BSe*RI) الذي اعطى قطع مختلفة الاحجام الجزيئية التي تعبر عن الانماط الوراثية.
أظهرت نتائج الدراسة ان (46%)من الرجال العقيمين هم ضمن الفئة العمريه (29-20) و(40%) ضمن الفئه العمريه (39-30) سنة بينما (68%) من النساء العقيمات ضمن الفئه العمريه (29-20).
بخصوص العلامات المناعية المختارة. وجد ارتباط معنوي في مصل الدم في مستوى الاجسام المضاده للهرمون المحفز للجريبات للنساء العقيمات (P0.32). ولم يظهر للجين protamine 1 اي علاقه بعقم الرجال .

Abstract
Background: Infertility is a worldwide problem which affects approximately 15% of all couples, and is defined as, inability to conceive after twelve months of contraceptive-free unprotected intercourse**. Materials and methods:** Optical density of anti follicle stimulation hormone antibody in the serum was detected by enzyme–linked immunosorbent assay technique and from which anti follicle stimulation hormone antibody concentrations were evaluated according to cut-off. One polymorphic sites of *Protamine 1 Gene* was genotyped on 72 couples suffering from infertility. Another group consist of 20 couples apparently healthy individuals. Genotypes were determined by the polymerase chain restriction fragment length polymorphism (PCR-RFLP) method. **Results:** Anti FSH antibody result demonstrated differences in antibody values between the patients and the control group with significant statistically, the frequency of the reported SNP was no significantly different compared to normal fertile males, which suggest that such SNP may not serve as a good molecular marker for genetic diagnosis of male infertility. **Conclusion:** The SNP G197T are completely absent and are not associated with male infertility with aforementioned, therefore these SNPs may not represent as a molecular marker for the diagnosis of genetic cause of male infertility in our studied population.
Keywords: Infertility; Genetic; anti-FSH antibodies, *Protamine 1 Gene*; Polymorphism; Genotype; Allele.

Introduction

There is no unanimous definition of female infertility, but nice guidelines state that: "A woman of reproductive age who has not conceived after 1 year of unprotected vaginal sexual intercourse, in the absence of any known cause of infertility, should be offered further clinical assessment and investigation along with her partner **(1)**. This public health problem involves all regions of the World. Its prevalence varies from 10 to 30 %, , with the man being responsible for nearly half of the cases despite advances in

assisted reproduction technologies making paternity possible for many of these men **(2).** Infertility could be as a result of anatomical, physiological, pathological, endocrinological, immunological and psychosexual character, immunoinfertility is one of the major causes of infertility in humans **(3).** The etiology of many cases of infertility remains poorly understood. It is obvious that infertility causes are heterogeneous because a number of factors contribute to reproductive success, about 30% of couples with reproductive problem are diagnosed with unexplained infertility **(4)**. Current evidence suggests that genetic factors contribute to the etiology of female infertility in humans **(5)**. Genetic factors influence all stages of development and functioning of the reproductive system. Genetic factors that may disrupt reproductive function include chromosome abnormalities (numerical and/or structural), mutations of genes controlling reproductive function (Y-linked, X-linked, autosomal, mitochondrial), mosaicism, different DNAs and chromosome polymorphisms as well as epigenetic factors **(6).**

The genetic causes of the majority of male infertility remains unknown, protamines are small basic proteins widely conserved among species, all mammals have one protamine, PRM1, while some species including human have a second protamine, PRM2. Because of the critical roles the protamines play in spermatid differentiation, aberrations in protamine expression or changes in protein structure could be causes of certain idiopathic human male infertilities **(7).**

DNA damage reduces fertility in female ovocytes and male sperm, as caused by smoking **(8).** Other xenobiotic DNA damaging agents such as radiation or chemotherapy **(9)** or accumulation of the oxidative DNA damage 8-hydroxy-deoxyguanosine **(10)**. And DNA damaging agents including reactive oxygen species, fever or high testicular temperature **(11).** Toxins such as glues, volatile organic solvents or silicones, physical agents, chemical dusts, and pesticides increased risk of infertility **(12).** Tobacco smokers are 60% more likely to be infertile than non-smokers **(13).** German scientists have reported that a virus called Adeno- associated virus might have a role in male infertility **(14)**. Follicular Stimulation Hormone (FSH) is one of the two pituitary gonadotrophins involved in regulating ovarian function, it has some influence on the development of follicles **(15).** Infertile women with anti-ovarian antibodies often display antibodies against FSH (anti-FSH) **(16).** It has been previously demonstrated that anti-FSH antibodies are elevated in infertile women **(17).**

The aim of this study is to investigate whether G197T of *PRM1* polymorphism might be involved in the pathogenesis of infertile males and whether there is the role of AFSH antibodies in infertile females**.**

Materials and methods

Subjects. The current study was conducted on 72 couples (72 female and 72 male) suffering from infertility for more than one year duration, seen in Maternity and Children Teaching Hospital and some Privet Clinic in Diwaniya city during the period from December 2012 to May 2013. Another group consist of 20 couples apparently healthy individuals (20 male and 20 female) without any history of systemic disease were clinically considered as healthy also included in this study as a control group. This study was in agreement with ethics of Al-Diwaniya Teaching Hospital and verbal informed consent was obtained from all participants..The patients were diagnosed clinically by physician as having infertility .

Blood samples for Anti FSH Antibody.

Three ml of venous blood samples were collected from both controls and couple patients who are suffering from infertility. A total of 144 blood samples were collected for detecting the anti FSH antibodies by using ELISA. Blood samples were collected by using a sterile syringe and put in a sterile clean glass test tube without anticoagulant. The blood left to clot for 30 minutes and then centrifuged at 3000 rpm. for 5 minutes. Serum sample was collected in plane tube and labeled for each patient. All serum

samples were frozen at -20c° until tested by ELISA kit.

DNA Extraction and Genotyping. Genomic DNA was extracted according to the manufacturer's protocol from 5 ml of frozen whole blood using a DNA Extraction Kit (Geneaid / USA). The polymorphic region was amplified by PCR. Amplification reaction were preformed in 0.2 ml tube of Accu Power PCR Premix tube according to the bioneer corporation then the thermocycling condition for this reaction carried out and products analyzed by 1% agarose gel electrophoresis **(18)**. PCR products were digested overnight with restriction enzymes (*BSeRI*), according to the manufacturer's protocol, and analyzed by 2% agarose gel electrophoresis.

Statistical analysis. Statistical analyses were done using SPSS version 20 computer software (Statistical Package for Social Sciences) in association with Microsoft Excel 2010. Odd ratio was used to measure the strength of association between 2 categorical variables and the statistical significance of the measured odd ratio is assessed by a special χ^2 formula. Deviations from Hardy-Weinberg equilibrium were investigated for all polymorphisms using the $\chi 2$ statistic, with expected frequencies derived from allele frequencies. An estimate was considered statistically significant if its P value was less than an α level of significance of 0.05.

Results

Subjects demographic characteristics.

Present study carried out on 72 couples (72 male and 72 female) seen in Al-Diwaniya Teaching Hospital, the age of the infertile males varied from 18 to 52 years with a mean age of 30.85 years (SD±7.17), compared with 20 healthy males with age range from 19 to 51 years, and mean age of 31.25 years (SD±7.85) as a control group, table (1).

Table (1):Distribution of infertile and control male according to the age.

	Study groups	
	Controls	Infertile patients
Age groups (male)	**N(%)**	**N (%)**
<20 years	1 (5%)	2 (3%)
20-29 years	9 (45%)	33 (46%)
30-39 years	7 (35%)	29 (40%)
40-49 years	2 (10%)	6 (8%)
≥50 years	1(5%)	2 (3%)
Total	**20 (100%)**	**72 (100%)**

Serum AFSH among Infertile and Control females.

The percentage of positive cases for (IgG) AFSH Antibody among serum of 72 infertile females was (18%), with (0%) among fertile females but with significant (p = 0.032), table (2).

Table (2): comparison of AFSH serum level in patients and controls (females).

	Study groups		Fisher's Exact Test
Serum AFSH	Control	Infertile patients	
	N (%)	N (%)	
Negative	20 (100%)	59 (82%)	0.032*
Positive	0 (0%)	13 (18%)	
Total	20 (100%)	72 (100%)	

*** = Significant difference (p≤0.05).**

Detection of *BseRI* Polymorphism:

Agarose gel electrophoresis image explain REFLP PCR product of protamine 1 gene in patient and healthy control samples, where, M: Marker 2000-100bp, Lanes (1-10): patient samples, and lanes: (11-15) healthy control samples; Restriction enzyme *BseRI* was cut the protamine 1 gene 557bp at one sit lead to two products 319bp and 238bp which mean no allele mutation detection., The mentioned SNP was not identified in the population screened in the present study. In all 92 samples (72 infertile and 20 fertile individuals) both of fragments which were products after *BSe*RI digestion, suggesting that there is no G197T SNP of *PRM1* in the studied population Figure (1).

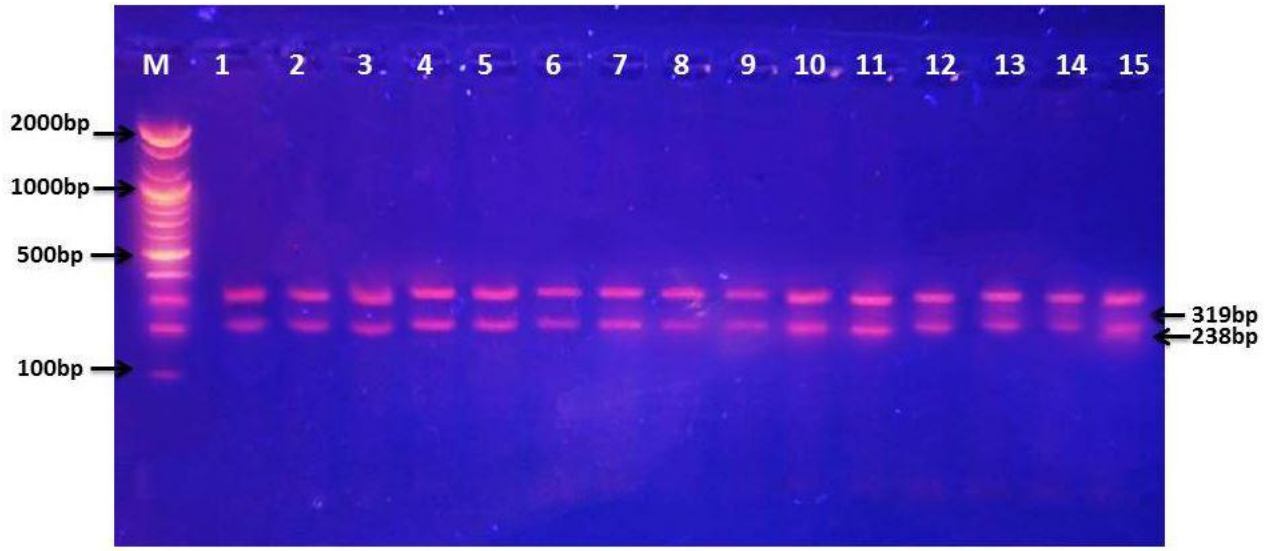

Figure (1):Ethidium bromide-stained agarose gel of PCR – RFLP amplified 238bp and 319bp of protamin 1 gene for study groups. Lane (M): DNA molecular size marker (KAPA Universal Ladder) , Lane 1-10 for infertile patients, Lane 11-15 for control group.

Discussion

The present study found the highest frequency of males with infertility was among the age of 20-29 years old (46%), followed by the age group of 30-39 years old (40%), this result agreed with results

of Mostafa,(2011), which found that the highest prevalence of infertile men at age <30 years, followed by age 30-34 years and decrease with age **(19).** Similar results were obtained from a study conducted in Iran which found that the majority of patients were within the age group of 20-29 years **(20).** And this results may be due to early marriage when the couples get married at young age and failed to conceive they seek treatment early even before a year. Other studies reported increase of infertile men at age of 26-35 years (63.50%) because that the youngest age groups are seeking more medical advices and they are more directed to fathering a child, in addition they are facing more psychological pressure and stress about their families, it was assessed that semen quality, frequency of ejaculation, and sperm functions gradually decrease with highly developed age and starts to decrease after 35 years **(21).** The pollutions, stress, and economic difficulties faced might be the main factors leading to infertility which affect this active age group **(22).** The differences in antibody values between the patients and the control group were statistically relevant but still rather small, meaning that these antibodies were also common among healthy women and could therefore merely represent examples of naturally occurring antibodies, these data support our previously published results Haller *et al.*, 2012 who found P <0.05 of study groups were compared with the controls and considered as statistically significant difference**(23).** The immune system must be altered in order to continuously produce anti-FSH antibodies, whether the antigenic FSH comes from seminal fluid or from the circulating hormone from the female organism, the idea of immune system alterations as well as the higher production of several types of autoantibodies in endometriosis and polycystic ovary syndrome **(17).** There is some indirect evidence that anti-FSH IgG antibodies may, however, further worsen female fecundity by reducing the FSH functionality, these data lead us to investigate the effect of anti-FSH antibodies on folliculogenesis and developing infertility in women **(23).** In addition to reflecting just immune system alterations, FSH-antibodies could also have an impact on the development of infertility, the serum anti-FSH antibodies were directed against the immunodominant epitope of the β -chain of the human FSH molecule, the 78–93 amino acid region , in this particular region, there is a loop called cysteine noose or determinant loop, the residues of which play a role in determining the specificity of FSH receptor binding **(17).** These data together indicate that infertility itself, rather than the cause of infertility, could be a predictive factor for the emergence of anti-FSH antibodies **(23)**.

This result was agreed with the report of **(24)** in Iran, who found in 308 samples (273 infertile and 35 fertile individuals) all PCR products of *PRM1* genomic fragments (557 bp) were used for digestion with *BSe*RI, the absence of mentioned SNP results in full enzymatic digestion of the amplified fragment, which produces two fragments with different length (238 bp and 319 bp) and there is no G197T SNP of *PRM1*.This result disagree with Iguchi *et al*.(2006) in USA **(7),** who found in 40 samples (30 infertile and 10 control individuals) one heterozygous single nucleotide polymorphism (SNP) at nucleotide 197 was identified in the DNA of three of the 30 infertile men examined, were seen in the three unrelated infertile men indicating a polymorphism of one *PRM1* allele. Based upon the absence of this SNP in this study, it appears to be a uncommon SNP **(18).** Addition to disrupting an arginine core essential for DNA binding, because SNP causes an amino acid change from arginine to serine in a highly conserved arginine

cluster **(7).** Indeed , evaluating two SNPs in *PRM1* in 1195 individuals, one of which was the same as the SNP assessed in this study and they concluded that this SNP has no significant effect in male infertility **(25).**

Conclusion

i. Female fertility can be affected by immune system.. Anti-ovarian autoantibodies are mostly directed against follicle stimulating hormone (anti-FSH).

ii. The SNP G197T are completely absent and are not associated with male infertility with aforementioned, therefore these SNPs may not represent as a molecular marker for the diagnosis of genetic cause of male infertility in our studied population.

iii. Analysis of the genetic factors that impact male factor infertility will provide valuable insights into the creation of targeted treatments for patients and the determination of the causes of idiopathic infertility.

Recommendations

For more precise results other more developed diagnostic tests like IF assay and PCR for detection of Anti FSH Antibody are recommended.

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