ABSTRACT

Single nucleotide polymorphisms are considered as one of causes of male infertility. The eNOS is primarily responsible that create of NO in the vascular endothelium, therefore eNOS is mainly expressed in the human testicular endothelial cells, in addition of Sertoli cells and Leydig, epididymis and vas deferens which regulates the synthesis of NO. Many study informed in the first time about the NO that is synthesized by human male gamete. Experimental evidence has shown that NO in high concentrations can cause defective function of the sperm and low concentrations of NO play very important role in the control of the sperm physiology. In this study, the relationship of eNOS polymorphism (rs1799983 T>A) in exon 8, was studied in 50 asthenozoospermic patients and 50 fertile men. Analysis of these SNPs was performed using real time thermocycler (Real-time PCR). As related with eNOS genes rs1799983T>A SNP, TT (homozygous) showed no significant differences in frequency percentage were noted between fertile men subjects and asthenozoospermia patients Whereas, the frequency of heterozygous TA genotype in asthenozoospermia patients are higher than fertile men group, but showed no risk factor. As related with AA (homozygous) showed a risk factor to cause infertility.

Keywords: eNOS gene, sperm, fertility, asthenozoospermia

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INTRODUCTION

Infertility is a major health problem worldwide, affecting at least one in every eight couples, and affecting people both medically and psychosocially (1). Five to 10% of normal fertile couples take more than a year or 2 to conceive. Some couples, therefore present with a delay in conceiving purely by chance, having normal low fertility rather than subfertility. Many of these supposedly “infertile” couples will eventually conceive, even without treatment. About 6% of men between the ages of 15 and 50 years are infertile. According to the World Health Organization (2), 60–80 million couples suffer from infertility worldwide (3). A male partner factor contributes to 40% of cases of infertility (4). In Iraq Infertility is a problem among men and women which considered as an important public health and clinical problem in Iraqis. In a study conducted in Iraq, (5) reported
incidence of primary infertility as 77.2 % and secondary infertility as 22.8 %; these percentages give a strong indication of infertility among Iraqi couples. Asthenozoospermia, a disorder of sperm motility, causes a reduced sperm motility; a reported study at a frequency of that 1 of 5000 men have 100% immotile spermatozoa in their ejaculate and there are many causes of asthenozoospermia include metabolic deficiencies and abnormalities of the sperm flagellum.

Asthenozoospermia, is a cause of human male infertility and is implicated in 19% of infertile cases. The methods for evaluation of male infertility have typically been limited to semen analysis measuring count, motility and morphology of the sperm. Up to 8% of infertile men have been shown to have high levels of sperm DNA fragmentation despite a normal semen analysis. Since sperm DNA has little capacity for repair, protection against damage is particularly important. The variants of the eNOS gene are involved in low spermatoogenesis and sperm function that be associated with man infertility in many studies of different populations like oligozoospermia and idiopathic AZS. The eNOS gene is a protein coding gene that is located in the 7q35-7q36 region in chromosome 7, that gene contains 25 introns and 26 exons with approximately 21kb length.

MATERIALS AND METHODS

Subjects and sampling
Blood and semen sample were collected from men with Asthenozoospermia patients (n=50) and fertile men as a control (n=50), were recruited from Kamal Al-Samraee hospital, Baghdad, Iraq. All infertile patients in this study were selected on the basis of clinical and laboratory examination. Blood samples were collected in EDTA K3 tubes from all infertile and fertile males in this study. The laboratory study was conducted in Genetic Engineering and Biotechnology Institute (GEBI), university of Baghdad, Iraq during a period from 15 November 2017 to 1 May 2018.

Analysis of gene
Genomic DNA was extracted from whole blood of infertile and fertile males using Wizard genomic DNA purification kit Geneaid (Bioneer). Real-time thermocycler (Real-time PCR) were performed using primers and probes that used to identify and amplify the 248 base pair fragment which include SNP, rs1799983, T894A, found in exon 8.

In Silico study
Many web servers were used for in silico prediction for Glu 298Asp SNP T894A (rs 1799983) in exon 8 of eNOS gene.

Statistical analysis
Comparisons of genotype and allele frequencies among study groups were determined using chi square test by

RESULTS AND DISCUSSION

Semen analysis
The diagnosis of male infertility is mostly based on the descriptive evaluation of human semen including the number of spermatozoa per ejaculate, sperm motility and their morphology \(^{14}\). Some semen parameters of fertile men and asthenozoospermia patients are present in table (1).

No significant differences were noted between asthenozoospermia patients and fertile men as related with age. The equal age allows correct comparison between the two groups as related with semen characteristic. Semen volume was significantly (p<0.05) increased in fertile men when compared with asthenozoospermic patients (2.66 versus 2.05 ml, respectively).

No significant differences were noted in semen pH between fertile men and asthenozoospermic patients group, pH values were within normal level in both two groups. Sperm count was in fertile men significantly (p<0.01) higher than that of asthenozoospermic patients (88.20 versus 45.33 \(10^6\)/ml, respectively). In both groups, sperm count was within normal values \(^2\). Sperm motility percentages was in asthenozoospermic patients significantly (p<0.01) lower than that of fertile men (8.60 versus 84.10%, respectively). Also, active sperms percentage was in asthenozoospermic patients significantly (p<0.01) lower than that of fertile men (1.20 versus 40.15 %, respectively). The results revealed that low sperm motility percentage in asthenozoospermic patients is accompanied with low active sperm percentage. \(^{14}\)Indicate that low sperm motility percentage led to impair male infertility.\(^{14}\)Observed that high percentage of progressively motile sperm in the ejaculate is critical to ensure adequate sperm transport and fertilization. The percentage of normal sperm were significantly (p<0.01) lower in asthenozoospermic patients than in the fertile men (6.80 versus 60.50%, respectively). In contrast, the percentage of abnormal sperm was in asthenozoospermic patients significantly (p<0.01) higher than that of fertile men (90.05 versus 33.11 %, respectively).

<table>
<thead>
<tr>
<th>Table 1: Compare between patients and fertile in seminal fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Seminal parameter</strong></td>
</tr>
<tr>
<td>(No. =50)</td>
</tr>
<tr>
<td>Age (year)</td>
</tr>
<tr>
<td>(35-50) 16</td>
</tr>
<tr>
<td>Volume (2-2.5ml)</td>
</tr>
<tr>
<td>pH (More than 7.2)</td>
</tr>
<tr>
<td>Count (More than 20 (10^6)/ml)</td>
</tr>
<tr>
<td>(More than 50%)</td>
</tr>
<tr>
<td>Motility (More than 5%)</td>
</tr>
<tr>
<td>Active (More than 15%)</td>
</tr>
<tr>
<td>Nor. Sperm (Less than 85%)</td>
</tr>
<tr>
<td>Abo. Sperm (Less than 85%)</td>
</tr>
</tbody>
</table>

* (P<0.05), ** (P<0.01). Control: fertile, Patient: asthenozoospermic, NS: Non-significant. **: means the difference is high significant at (p<0.01),

In silico matching of primers and probes

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The sequence of primers and probes were matched using FASTA format by National Center for Biotechnology Information (NCBI). The wild type detecting probe was labeled with FAM in the 5’ end and Minor groove binder probe (MGB) in the 3’ end. The probe prepared for the mutant allele (SNP) was labeled with VIC in the 5’end and MGB in the 3’end for eNOS polymorphism (rs1799983 T>A) as shown in figure (1).

**Figure 1**: Matching of the primers and probes sequences for eNOS polymorphism (rs1799983 T>A) on the bioinformatic programs/ NCBI

**Figure 2**: The Homozygous mutant genotype. The photograph was taken directly from Agilent qPCR machine
A fragment in eNOS gene was amplified as a 248 bp fragment (T894A). The Real time thermocycler condition for amplification of eNOS gene fragment T894A was as, in cycling 1 is 5 cycles of denaturation at 95°C for 5 second, annealing at 60°C for 20 second, and extension at 72°C for 15 second, and in cycling 2 is 40 cycles of denaturation at 95°C for 5 second, annealing at 60°C for 20 second, and extension at 72°C for 15 second. In this step the acquiring Green and Yellow (FAM and VIC) were added.

The eNos gene rs1799983 T>A Polymorphism

In silico study

Many web servers used for in silico for rs1799983 T>A of eNOS gene.

PROVEAN

In this study, the SNP rs1799983 of eNOS gene was subjected to PROVEAN (provean.jcvi.org/seq-submit.php). The provean score of rs1799983 SNP of eNOS gene was -0.900 then the prediction of this variant was deleterious because the score is below -2.5 as shown in figure (5).
Figure 5: The PROVEAN prediction for the effect of the SNPT894 A(rs 1799983)eNOS gene

**Polyphen-2**

The PolyPhen-2 score can be interpreted as follows: 0.0 to 0.15: Variants with scores in this range are predicted to be benign. 0.15 to 1.0: Variants with scores in this range are possibly damaging. 0.85 to 1.0: Variants with scores in this range are more confidently predicted to be damaging. The PolyPhen-2 score of the 1799983 SNP of eNOS gene was 0.512 therefore this mutation is predicted to be possibly damaging as shown in figure (6).

Figure 6: Prediction possible impact of the SNPT894 A(rs 1799983)eNOS gene using PolyPhen-2.
In this study, the rs1799983 SNP of eNOS gene was subjected to I-Mutant 2.0. The DDG (Reliability index) of rs1799983 SNP of eNOS gene was equal to 5 than the predicted protein stability of this variant was decrease because the RI is more than zero as shown in figure (7).

Figure 7: Prediction possible impact of the SNPT894 A (rs 1799983) eNOS gene using I-Mutant 2.0.

PhD-SNP
As shown in figure 4-8, the rs1799983 SNP of eNOS gene was is disease – related polymorphism. The value of sequence and profile – based prediction was equal to 4.

Figure 8: The predicted deleterious of eNOS gene as affected by the SNPT894 A (rs 1799983) using PhD-SNP.
Molecular study
This SNP is located in exon 8. The distribution of genotypes and allele frequency at rs1799983 SNP of eNOS gene for Iraqi fertile men and asthenozoospermic patients presented in table (2).
As related with homozygous TT genotype, the frequency was significantly (p<0.01) lower in patients with asthenozoospermia than fertile men (control) (0% versus 36%, respectively, $\chi^2 = 9.82$, p<0.01).
The frequency of TA genotype was in asthenozoospermic patients significantly (p<0.05) higher than in control fertile men (70% versus 62 %, respectively, $\chi^2 = 4.31$, $OR = 0.662$, p<0.05).
Also the frequency of AA genotype was in asthenozoospermic patients significantly (p<0.01) higher than in control fertile men (30% versus 2 %, respectively, $\chi^2 = 9.07$, $OR = 1.26$, p<0.01). As shown from the results, AA genotype was appearing to be a risk factor for asthenozoospermia in the present study.
The frequency of T allele was 0.67 and 0.35 in fertile men and asthenozoospermic patients respectively. Also the frequency of A allele was 0.33 and 0.65 in fertile men and asthenozoospermic patients respectively.

Table 2: The frequency of genotypes and alleles at rs1799983 SNP of eNOS gene in Iraqi men with Asthenozoospermia and controls

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Frequency,n(%)</th>
<th>$\chi^2$</th>
<th>OR $^3$</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control$^1$</td>
<td>Patients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>18(36%)</td>
<td>0(0%)</td>
<td>9.82 **</td>
<td>--</td>
</tr>
<tr>
<td>TA</td>
<td>31(62%)</td>
<td>35(70%)</td>
<td>4.31 *</td>
<td>0.662</td>
</tr>
<tr>
<td>AA</td>
<td>1(2%)</td>
<td>15(30%)</td>
<td>9.07 **</td>
<td>1.26</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Alleles frequencies</th>
</tr>
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<tbody>
<tr>
<td>T</td>
</tr>
<tr>
<td>0.67</td>
</tr>
<tr>
<td>0.35</td>
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<td>---</td>
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</table>

<p>| A                   |
| 0.33                |</p>
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<th>0.65</th>
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</table>

$^1$ apparently healthy subjects. $^2$ Patients with Asthenozoospermia. $^3$ Odd ratios.

*: Significant at 0.05 level **: Significant at 0.01 level.

The results of in silico study on the rs1799983 SNP of eNOS gene confirm the molecular results on the same SNP in the present study. Using PROVEAN tool, the prediction of rs1799983 was deleterious.

In PolyPhen-2 tool, rs1799983 is predicted to be possibly damaging. In addition, I-Mutant 2.0 predicted protein stability of rs1799983 was decrease. The PhD-SNP predicts the rs1799983 SNP to be disease-related polymorphism.

These results are in agreement with many previous studies (16-18). (16) found a significant difference in frequency of the missense (rs1799983) variant of the eNOS gene between asthenozoospermic patients and the control group in Italian population, (17) found that AA genotype of rs1799983 was significantly more frequent in infertile

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subjects. (18) revealed that eNOS rs1799983 was associated with an increased risk of male infertility in Chinese population.

Also, the results of rs1799983 of eNOS gene in this study are disagree with other previous studies (19,20). (19) Reported no significant association between control and infertile group at rs1799983 of eNOS gene in Korean population (20) found that the rs1799983 of eNOS gene was not associated with male infertility in Brazilian population.

The eNOS A allele may be associated with an increased production of NO that compromises sperm motility. The presence of the A allele may be the cause of altered eNOS protein structure and function that increases the incidence of oxidative stress-induced sperm damage in infertile men, leading to asthenozoospermia (21). (22) Indicated that the A allele of this missence eNOS SNP is associated with high NO levels in healthy people.

As related with eNOS protein, (23) indicated that rs1799983 has a functional effect on the eNOS protein. While, (24) found that the A allele of this missence eNOS SNP is associated with eNOS protein levels.

As related with sperm motility, (16) found an association between rs1799983 SNP of eNOS gene and sperm motility in asthenozoospermic patients and that A allele of rs1799983 contributed to poor sperm motility. Also, they found that the asthenozoospermic patients who had a wild type (TT) genotype had significantly higher values of sperm motion kinetics than patients with a heterozygous (TA) or homozygous (AA) genotype.

The eNOS rs1799983 variants may have higher eNOS levels and activity, resulting in a high concentration of NO; a high NO concentration may cause sperm DNA damage, thereby contributing to male infertility (18).

Computer analysis has revealed that the rs1799983 of eNOS gene results in a conformational change in the eNOS protein from a helix to a tight turn and may affect the protein-protein interactions and localization of the eNOS protein, which might also affect the protein function and explain the enhanced disease risk associated with the presence of rs1799983 polymorphism in the eNOS protein (25).

CONCLUSION

In eNOS gene, AA genotype of T894Ars1799983 SNP showed a risk factor to cause infertility asthenozoospermia, in Iraqi patients.

ETHICAL CLEARANCE

The Research Ethical Committee at scientific research by ethical approval of both environmental and health and higher education and scientific research ministries in Iraq

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

FUNDING: Self-funding
REFRENCES


