Endothelial Nitric Oxide Synthase (eNOS) Gene Polymorphism, Nitric Oxide and Progesterone Levels in Unexplained Recurrent Pregnancy Loss

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Abstract: This study was conducted in order to determine the relationships between promoter -786 T>C, exon 7 Glu298Asp (894 G>T) and intron 4 (4a4b) VNTR polymorphisms of eNOS gene, serum nitric oxide (NO) and progesterone (P4) levels, and unexplained recurrent pregnancy loss (URPL) in Palestinian women residing in Gaza strip. The study presented here is an association study with a case-control design. The study population consisted of 45 (30 non-pregnant and 15 pregnant) women who suffered from URPL, and 45 (30 non-pregnant and 15 pregnant) healthy women matched for age and without a previous history of RPL. Two blood samples were collected from each subject after fasting for 10-12 hours, one was whole blood and the other was serum. DNA extracted from blood samples of all subjects was investigated for the three eNOS polymorphisms using PCR or PCR-RFLP. Serum NO level was measured using a colorimetric method. Serum progesterone was measured using Immulite 1000 Analyzer. The study showed that the promoter -786T>C polymorphism C allele (represented by CC + CT genotypes) is significantly associated with increased risk of RPL, where it occurred with a higher frequency in URPL women and was associated with decreased serum NO levels in this group (all P-values <0.001). Neither exon 7 Glu298Asp (894G>T) nor intron 4 (4a4b) VNTR polymorphisms was significantly associated with RPL risk in the study population. The serum NO levels were lower in URPL patients as compared to their respective controls (P-value =0.004). The study also pointed to a positive proportional correlation between serum nitric oxide and progesterone levels in the study population (P-value= 0.002, correlation coefficient= 0.319) that might be attributed to the presence of progesterone receptor binding sites in the eNOS gene. The C allele of the promoter -786T>C polymorphism is a possible risk factor for URPL in Gaza strip. Moreover, this same allele is associated with a decreased serum NO level that, in turn, is associated with URPL. Stemming from the observed correlation between serum NO and progesterone, we suggest that balancing progesterone and NO levels may be of benefit for maintaining a healthy pregnancy at least in some URPL cases.

Keywords: eNOS gene polymorphism, URPL, Nitric oxide, Progesterone, Gaza Strip, Palestine.

Introduction

Recurrent pregnancy loss (RPL) is defined as three or more consecutive pregnancy losses before 20 weeks of gestation [1]. RPL may affect 0.5–2% of women of reproductive age [2]. URPL is the RPL where the standard investigative protocol fails to identify the cause of losses. Nitric oxide (NO) was first recognized in the reproductive system by Ignarro et al. [3]. Later on it has been shown by other investigators abnormal levels of NO are involved in preeclampsia [4, 5] and, in malfunctioning of placental vasculature that may lead to various gestational complications [6]. Moreover, Yallampalli and Garfield have observed that inhibition of NO synthesis in rats during pregnancy produces hypertension, proteinuria, thrombocytopenia, and fetal growth retardation [7].

Nitric oxide synthase (NOS) enzymes are expressed in three isoforms: neuronal (nNOS), inducible (iNOS), and endothelial (eNOS). Endothelial nitric oxide synthase is the main enzyme required for vascular NO production [8]. The eNOS gene was first cloned in 1993 and was localized to chromosome 7q35-36 [9]. Several polymorphic variations of the eNOS gene have been identified, and many of them particularly, promoter -786 T>C, exon 7 Glu298Asp (894 G>T) and intron 4 (4a4b) VNTR, have been studied with regard to their association with URPL. Although some studies have demonstrated significant relationship between a particular polymorphism and increased risk of RPL in certain ethnic groups, many of the reported associations, however, have not been reproduced in other different ethnic groups [6, 10]. Additionally, the 4a/4b VNTR polymorphism has been shown to segregate with lower plasma NO metabolites [11].

Progesterone (P4) is essential for endometrial receptivity and successful establishment of pregnancy. Either an insufficient P4 concentration or an insufficient response to P4 can lead to infertility and pregnancy loss [12]. Progesterone also can increase uterine quiescence by stimulating the relaxation mechanisms, mainly the uterine NO system [13]. The present study is the first to evaluate the three eNOS gene polymorphisms
and their relation to serum NO and P4 levels and URPL in Gaza strip.

Materials and Methods

Study population:
The study was performed on 45 (30 non-pregnant and 15 pregnant) Palestinian women aging 18–35 from Gaza strip who had at least three URPLs ≤20 weeks of gestation. Age and ethnicity matched 45 (30 non-pregnant and 15 pregnant) women with at least two live births and without a previous history of abortion or pregnancy-associated complications served as the control group. Informed consent was obtained from all participants, and approval for conducting the study was obtained from the local ethics committee.

Blood collection:
Eight milliliters of venous blood were collected from each overnight fasting participant into one EDTA-anticoagulant and one Plain tube, under quality control and safety procedures. Blood in Plain tubes was used on the same day for serum preparation. Serum was stored at -80°C till analyses.

DNA extraction and polymorphism determination:
Genomic DNA was extracted from the EDTA-blood using Wizard Genomic DNA purification Kit (Promega, USA) following the manufacturer’s instructions. Genotyping was performed using polymerase chain reaction (PCR) and restriction length fragment polymorphism (RLFP). Primers and restriction enzymes described by Shin et al [8] were employed. All PCR reactions were carried out in a volume of 20µl containing 2µl of 10µM forward primer, 2µl of 10µM reverse primer, 4µl Nuclease free water, 10µl PCR Mastermix (Promega, USA), and 2µl (about 30 ng/µl) genomic DNA. The thermal cycling consisted of an initial denaturation at 94°C for 5 min, 35 cycles (94°C for 1 min, annealing at 58°C for 45 sec, and elongation in 72°C for 45 sec) and a final elongation at 72°C for 7 min.

The eNOS promoter -786T>C polymorphism was defined by digesting the PCR product with the restriction endonuclease NgoMIV (New England BioLabs) at 37°C for 16 hrs. The wild type -786T allele PCR product remains uncut (236-bp) whereas, the polymorphic -786C allele is cut into two fragments of 203- and 33-bp. The eNOS exon 7 894G>T polymorphism was determine by digesting the PCR product with the restriction endonuclease MboI (New England BioLabs) at 37°C for 16 hrs. The wild type 894G allele remains uncut (206-bp) while the polymorphic 894T allele is cut into two fragments of 119-and 87-bp. The amplified DNA fragments of the eNOS intron 4 VNTR 4a4b polymorphism were analyzed without digesting the PCR product. The wild-type allele (allele 4b) generates a 420-bp band (five copies of a 27-bp repeat) while, the polymorphic allele (allele 4a) generates a 393-bp band (four copies of the same repeat).

Measurement of serum NO Levels:
Serum NO levels were measured using a Nitric Oxide Colorimetric Kit (BioVision, USA) according the instructions of the manufacturer.

Measurement of serum P4 Levels:
Serum P4 levels were on Immulite 1000 Analyzer using Immulite®/Immulite® 1000 Progesterone kit (IMMULITE, USA).

Statistical analysis:
Statistical significance of the differences between the patient and control groups were estimated by Chi (X2) square test and independent samples t-test. Odds Ratio (OR) and 95% confidence intervals (CI) were analyzed by Fisher’s exact test. Statistical significance was set at P-value <0.05.

Results

Association between eNOS gene polymorphism and URPL:
Genotype and allele frequencies of promoter -786 T>C were significantly different between URPL patients and controls. On the other hand, neither genotype nor allele frequencies of exon 7 Glu298Asp (894 G>T) and intron 4 (4a4b) VNTR were significantly different between the two groups (Table.1).

Association between serum NO levels and RPL regardless of the eNOS polymorphism:
Table 3 illustrates the results of mean NO level in pregnant/non-pregnant URPL and their respective controls. A statistically significant difference was observed between the two study groups as whole and between non-pregnant URPL patients versus non-pregnant controls. A lower NO level, though not significant, was also observed in the pregnant URPL patients as compared to pregnant
controls.

### Table 1: Frequency of the eNOS gene polymorphisms among URPL patients and control subjects

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Genotype/Allele</th>
<th>URPL (N=45)</th>
<th>Control (N=45)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promoter</td>
<td>-786 T&gt;C</td>
<td>TT</td>
<td>53.3% 86.7%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>N (%)</td>
<td>CC + CT</td>
<td>46.7% 13.3%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>T allele</td>
<td>73.3% 93.3%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C allele</td>
<td>26.7% 6.7%</td>
<td></td>
</tr>
<tr>
<td>Exon 7 (894 G&gt;T) N (%)</td>
<td>GG</td>
<td>T allele</td>
<td>57.5% 48.9%</td>
<td>0.398</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G allele</td>
<td>75.6% 70.0%</td>
<td>0.402</td>
</tr>
<tr>
<td>Intron 4 (4a4b) VNTR</td>
<td>4a4b</td>
<td>T allele</td>
<td>24.4% 30.0%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4b allele</td>
<td>95.6% 100%</td>
<td>0.153</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4a allele</td>
<td>4.44% 0.0%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4b allele</td>
<td>97.8% 100.0%</td>
<td>0.155</td>
</tr>
</tbody>
</table>

### Table 2: Difference in the mean levels of NO with respect to eNOS polymorphisms

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Genotype</th>
<th>NO concentration Mean ± SD (µM)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promoter</td>
<td>-786 T&gt;C</td>
<td>TT 19.3 ± 6.39</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>N (%)</td>
<td>CC + CT 9.2 ± 2.16</td>
<td></td>
</tr>
<tr>
<td>exon 7 (894 G&gt;T) N (%)</td>
<td>GG</td>
<td>TT + GT 17.4 ± 8.17</td>
<td>0.096</td>
</tr>
<tr>
<td>Intron 4 (4a4b) VNTR</td>
<td>4a4b</td>
<td>4b allele 16.2 ± 0.75</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>4a allele</td>
<td>19.2 ± 11.20</td>
<td></td>
</tr>
</tbody>
</table>

### Table 3: Difference in the mean level of nitric oxide between URPL and control

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean ± SD (µM)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>URPL (pregnant + non-pregnant)</td>
<td>45</td>
<td>14.17 ± 6.95</td>
<td>0.004</td>
</tr>
<tr>
<td>Control (pregnant + non-pregnant)</td>
<td>45</td>
<td>18.40 ± 6.73</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>non-pregnant URPL</td>
<td>30</td>
<td>11.54 ± 4.08</td>
<td></td>
</tr>
<tr>
<td>non-pregnant Control</td>
<td>30</td>
<td>15.52 ± 4.47</td>
<td></td>
</tr>
<tr>
<td>Pregnant URPL</td>
<td>15</td>
<td>19.42 ± 8.56</td>
<td>0.11</td>
</tr>
<tr>
<td>Pregnant Control</td>
<td>15</td>
<td>24.13 ± 6.94</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Non-pregnant Control</td>
<td>30</td>
<td>15.52 ± 4.47</td>
<td></td>
</tr>
<tr>
<td>Pregnant Control</td>
<td>15</td>
<td>24.13 ± 6.94</td>
<td></td>
</tr>
</tbody>
</table>

Relation between NO and progesterone

Results showed the presence of a significant positive correlation between serum NO and P4 levels in the study population (P-value= 0.002, correlation coefficient= 0.319). Moreover, no significant association was observed between the -786 T>C polymorphism and serum P4 levels. The mean serum progesterone level in the C-allele carriers was 6.56ng/ml as compared to 8.58ng/ml in the TT-genotype women (P-values=0.401).

### Discussion

The current study was designed to investigate the association between three eNOS gene polymorphisms and URPL in Gaza Strip-Palestine.

#### Association between eNOS gene polymorphisms and RPL:

The study indicated lack of significant association between URPL and two of the investigated polymorphisms; exon 7 Glu298Asp and intron 4 (4a4b) (Table 1). Similar results were reported for women from Greek [11], Indian [14], and Tunisian [15] populations. In the contrary, results for women from other populations indicated that one or the other of those polymorphisms is significantly associated with RPL [16].

Regarding intron 4 (4a4b) polymorphism, our results showed that both the 4a/4b genotype and the 4a allele were encountered in only 2 cases which belonged to the URPL group. This result indicates that the 4a allele is not common in our population and may explain why the 4a/4a genotype was not encountered in any of the subjects enrolled in the study. This finding supports the earlier results of Sallout and Sharif where they also found that the 4a allele is not common in our population [17]. A very low frequency of "4a" allele was also observed in some other populations [16]. Still, the role of the "4a" allele in RPL should not be neglected since this allele was found only in URPL subjects both in the current study and in the study of Sallout and Sharif [17]. Therefore, studies on larger samples are needed in order to verify this point.

A handful of studies have investigated the relation between eNOS -786T>C polymorphism and pregnancy complications (including RPL) in women from various populations [8]. Our results for this particular polymorphism showed significant association between the C-allele carriers (both CC and CT genotypes) and URPL. This result is consistent with that published by Shim, et al. (2010) [18] and those reported for Caucasian women of Polish origin [19]. In contrast, our results do not support the previously published results for women from Korean [8] and Tunisian [15] populations which indicated that promoter -786 T>C polymorphisms is not significantly associated with RPL.
The very different outcomes of RPL genetic association studies may be attributed to differences in genetic background and gene environment interactions among various populations. Moreover, the small number of patients (due to the low incidence of URPL) recruited in such studies is another important determinant. The present data add to the importance of ethnic as well as intra-regional variability in studies concerning multifactorial disorders including URPL. Our findings regarding the three investigated eNOS polymorphisms, however, clearly showed that the promoter -786T>C polymorphism of the eNOS gene, namely "allele -786C" is associated with RPL in Palestinian women residing in Gaza strip.

Association between eNOS polymorphisms and serum NO levels:

The present study showed that promoter -786 T>C polymorphism is significantly associated with low serum NO levels (Table-2). This is in agreement with the finding of Dosenko et al who reported that promoter -786 T>C polymorphism is associated with low eNOS gene promoter activity in platelets [20], reduced placental eNOS mRNA levels, and low serum nitrite/nitrate levels [21].

The association between the -786C allele and reduced NO level points to the presence of a binding site for a transcription factor and to the critical role of this particular nucleotide. In fact, reporter gene studies have shown that promoter -786T>C substitution markedly blunts the transcription rate of the eNOS gene, and hence NO production, likely because the C allele creates a binding site for a replication protein A1 (RPA1) that acts as a suppressor of eNOS transcription. Furthermore, it has been shown the level of eNOS mRNA in placentas with promoter -786T>C substitution mutation (i.e., C-allele) is significantly lower than in placentas without the mutation [21]. These findings confirm our results that some URPL women are associated with a high frequency of promoter -786T>C polymorphism C allele and might explain why this polymorphism is associated with a low serum NO levels.

Association between serum NO levels and RPL regardless of the eNOS polymorphisms:

The lack of a significant difference in the mean NO level between pregnant URPL patients versus pregnant controls, may be attributed to the small number of the two groups which consisted of only 15 subjects each. Though, not significant, it should be emphasized here that the mean NO level (19.42 µM) in the pregnant RPL group was clearly lower than its level in the pregnant control subjects (24.13 µM).

Several studies have been concerned with the role of NO in RPL, but their findings were contradictory. Our result is in agreement with those of Baban et al., (2010) where they found that serum NO levels in RPL patients showed a highly significant decrease compared with third trimester pregnant, and non-pregnant control women. They also reported that the decrease in NO production is a result of RPL and not a causative factor [22]. Paradisi et al (2007) reported that serum NO levels in the missed abortion group were extremely significantly lower than both the non-pregnant and the pregnant control groups [23]. Delacretaz et al., (2005) suggested that NO synthesis is increased significantly during normal pregnancy, possibly contributing to the vasodilatation. While, NO generation, may be inappropriately low in pregnant women developing preeclampsia, thus leading to an enhanced vasoconstriction [24]. Thanda et al., (1996) showed that the NOS activity is highest in the early gestational age placenta, suggesting a possible significant role of NO in early gestation [25]. Our results also support the findings of Wilson et al (1997) where they reported that serum NO levels are significantly lower in the non-pregnant URPL group than those in the non-pregnant control group [26]. (Table.3), indicating that a genetic factor rather than pregnancy itself is responsible for the low NO level.

Given the vasodilation nature of NO we believe that normal pregnancy phases should be associated with particular levels of NO and that imbalances in those levels can lead to adverse outcomes such as preeclampsia and fetal loss.

The positive correlation between NO level and P4 observed in this study is in harmony with many earlier studies, which all proposed that P4 can up-regulate eNOS protein expression in the myometrium [27] and in turn stimulate NO production, both by non-genomic and genomic mechanisms [28]. The non-genomic mechanism is executed through a rapid signaling mechanism involving activation of a membrane bound receptor and subsequent activation of mitogen-activated protein kinase (MAPK) and PI 3-kinase/Akt pathways resulting in eNOS phosphorylation and increased eNOS activity [28, 29]. The genomic mechanism is assumed to be through increase in eNOS mRNA and subsequent NO production [29]. By in silico analysis of the eNOS gene sequence we detected five possible progesterone receptor binding sites, which all have the canonical progesterone receptor binding sequence: "5'-TGTCTT-3'" [30].
Two of these putative binding sites are located at (4138 bp) and (2246 bp) up-stream the translation start site (TSS). The remaining 3 sites are located in introns 8 and 11, and (21765 bp) down-stream the TSS. We therefore assume that at least some of these sites could explain the correlation observed between P4 and NO levels, and work is ongoing in order to confirm this point.

**Association between eNOS promoter -786 T>C polymorphism and serum progesterone levels:**

The study showed lack of significant association between the eNOS promoter -786 T>C polymorphism and serum progesterone level. This finding may indicate that the C allele and P4 are lowering NO by two different mechanisms, as described above.

In conclusion, the C allele of the promoter -786T>C polymorphism is a possible risk factor for URPL in Gaza strip. Moreover, this same allele is associated with a decreased serum NO, and stemming from the observed correlation between serum NO and progesterone, we suggest that lowering NO by two different mechanisms, as described above.

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**References**


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