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The Association of Paraoxonase 1 (PON-1) Gene Polymorphism at rs854560 SNP (L55M) and Some Oxidative Stress Parameters in Serum with the Incidence of Recurrent Spontaneous Abortion in Iraqi Women

[1]Zainab T. Hussein, [2]Ismail A. Abdul-Hassan

[1]Environment and Water Directorate, Ministry of Science and Technology
[2] Institute of Genetic Engineering and Biotechnology for Postgraduate studies, University of Baghdad

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Abstract: Recurrent spontaneous abortion (RSA) is characterized by the occurrence of two or more spontaneous pregnancy losses before reaching the midpoint of gestation. Oxidative stress assumes a central role in the development of RSA, serving as a key factor in its pathogenesis. The objective of this study is to explore the link between the L55M polymorphisms found in the PON1 gene, which codes for an antioxidant enzyme, and the susceptibility to RSA.Paraoxonase 1 (PON1) is a critical enzyme in the body's antioxidant defense system, safeguarding cells against damage caused by reactive oxygen species. The most prevalent PON1 polymorphism, L55M (rs854560), is known to influence PON1 activity. In this research, blood samples were collected from two groups: 50 women with a history of RSA and 50 women who were apparently healthy. Following DNA extraction, Restriction Fragment Length Polymorphism (RFLP) analysis was employed in conjunction with PCR amplification to identify single-nucleotide polymorphisms (SNPs) and ELISA for determining PON1 activity, total antioxidant capacity (TAC) and HDL concentration in serum. The result of this study showed decreasing in PON1 activity (8.15 ± 2.15 versus 9.66 ± 3.75 , respectively) and TAC (2.80 ± 0.10 versus 3.53 ± 1.52, respectively) in patients with RSA than apparently healthy women, while HDL concentrations higher in women with RSA than apparently healthy women (12.68 ± 5.53versus 9.01 ± 5.30, respectively) which are lead to increase oxidative stress and related with RSA. The genotype results showed that the percentage of TA genotype was in women with recurrent spontaneous abortion significantly higher than that of apparently healthy women (60% *versus* 32 %, respectively, OR=3.5) and this represent a risk factor for recurrent spontaneous abortion incidence. Also, the percentage of AA genotype in women with recurrent spontaneous abortion was higher than that of apparently healthy women but non-significant (12% and 4% 68%, respectively). The values of allele frequency for T allele were 0.8 and 0.6 for apparently healthy women and women with recurrent spontaneous abortion, respectively. The values of allele frequency for A allele were 0.2 and 0.4 for apparently healthy women and women with recurrent spontaneous abortion, respectively. It was concluded the heterozygous TA genotype is related with the risk of RSA and the minor allele (A allele) increased in women with RSA.

Keywords: Recurrent spontaneous abortion, PON1enzyme, L55M, oxidative stress,

Corresponding author: (Email: zainabtalal20@yahoo.com).

Introduction

Recurrent pregnancy abortion (RSA), which may occur twice or more, is the loss of the fetus before the 20th day of pregnancy. It is a complex disorder with many different etiological

variables (1). It is estimated that between 30 and 50 percent of conceptions go undetected in the first trimester of pregnancy. The majority of miscarriages happen around the time of implantation and can go unrecognized by the pregnant women themselves since they are frequently confused with delayed periods. Additionally, during the first trimester, between 10 and 12% of all clinically confirmed pregnancies result in miscarriages. Recurrent spontaneous aborters can be categorized into two main groups: primary recurrent spontaneous aborters and secondary recurrent spontaneous aborters. Primary recurrent spontaneous aborters refer to individuals who have encountered pregnancy loss in all their previous pregnancies and have not yet achieved a successful live birth. On the other hand, secondary recurrent spontaneous aborters are individuals who have had at least one successful pregnancy, regardless of the number of pregnancy losses they may have experienced (2). Approximately 50% of miscarriages are attributed to chromosomal abnormalities, while the remaining 50% are attributed to various factors, including anatomical, genetic, endocrine, immunological, and environmental variables. Multiple genetic mechanisms can contribute to infertility and various pregnancy complications. These mechanisms can lead to DNA damage and reduce the viability of both the embryo and the placenta, potentially resulting in pregnancy loss (3).In spite of that ,50% of couples still don't have an RSA diagnosis after a thorough evaluation (4).

Studies have suggested that oxidative stress and a decrease in the body's antioxidant capacity may be key factors in pregnancy-related illnesses like RSA (5). When present at very low levels, ROS activated molecular species resulting from oxygen metabolism play a pathogenic role in cell function in addition to a physiological one. Cells have developed antioxidant mechanisms to stop ROS caused damage. In order to maintain a physiological equilibrium leading to cellular homeostasis, there is a delicate balance between ROS generation and antioxidant activity. Cell damage and dysfunction occur when there is an imbalance caused by an excessive production of reactive oxygen species (ROS), leading to a state of oxidative stress (6).

PON1, GPX, CAT, and Superoxide dismutase are only a few of the numerous free radical inhibition systems found in the human body. PON1 is associated with high-density lipoprotein and is in charge of neutralizing free radicals soluble in lipid peroxidation lipids as well as detoxifying organophosphoric chemicals (7). Three PON1, PON2, and PON3 genes make up the paraoxonase gene family in mammles. All three genes' offspring have been found to have antioxidant activity, however PON1 plays the most antioxidant role. PON1 is a protein that weighs between 45 and 43 kDa and has 345 amino acids. The PON1 gene's two most significant polymorphisms are Q192R (rs662), which substitutes glutamine for arginine, and L55M (rs854560), which substitutes leucine for methionine (T >A) at the position 55 in exon 3 affects PON1 mRNA, protein level and its activity (8). Free radical neutralization and oxidative stress suppression are the primary and crucial functions of the PON1 enzyme. Together with other enzymes and antioxidants, this enzyme defends against oxidizing chemicals (9).

This study examines the relationship of pon1 gene

polymorphism at M55L SNP and some oxidative stress parameters with the risk of RSA in Iraqi women.

Materials and methods

Participants

This study is conducted in the obstetrics and gynecology department of El-Elwiya Teaching Hospital and the Laboratory of Institute of Genetic Engineering and Biotechnology for Postgraduate Studies University of Baghdad. The patients group included 50 females with recurrent spontaneous abortion (more than 3 abortion) during first trimester (10-12 weeks) . The criteria applied guarantee that the patient didn't suffer diabetes mellitus, hypertension, fever, thyroid disease, infection, anatomical disorder and any chronic disease cause abortion and the patients weren't alcohol, smoking and drugs consumption. Patients aged (25-45) years. The body mass index for each patient was calculated and the numbers of living child and abortion were accounted. The control group consisted of 50 pregnant women whose ages were matched with those in the patient group. These women were in apparent good health, had experienced at least two prior successful deliveries, and had no history of miscarriage.

Genetic analysis

For genotype analysis, genomic DNA was extracted from the whole blood sample by using *EasyPure*® Genomic DNA Kit (TransGen, biotech. EE101-01).The primers used for flanking the L55M SNP of pon1 gene was forward 5- CCTGCAATAATAT GAAACAACCTG-3 and reverse 5-TGAA AGACTTAAACTGCCAGTC-3 (10). Polymerase Chain Reaction (PCR) was conducted within a 25-µl reaction tube. In each reaction mixture, 2 µl of DNA, 1 µl of each primer, 12.5 µl of the master mix supplied by Amplicon, Denmark, and 8.5 µl of nuclease-free water were carefully blended together. The PCR cycling protocol initiated with an initial denaturation step at 94°C for 5 minutes, followed by 30 cycles, with each cycle encompassing denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute. Following PCR amplification, we conducted a Restriction Fragment Length Polymorphism (RFLP) analysis to identify single-nucleotide polymorphisms (SNPs). To process the PCR products, 0.5 µl of Hin1II enzyme was added to 10 µl of the PCR product, along with 2 µl of enzyme buffer and 7.5 µl of nuclease-free water. Following the combination of this mixture, it was then incubated at 37°C for 15 minutes to activate the enzyme, and subsequently subjected to a deactivation step at 80°C for 20 minutes. The enzyme employed in this process was Hin1II, and we utilized the FlyCut Hin1II Kit from TransGen Biotech (Catalog number JH101-01). After digestion, we performed electrophoresis using 2.5% agarose gels to separate the fragments, followed by staining for detection.

Determination of PON1activity, TAC and HDL in serum

The measurement of PON1 enzyme activity, total antioxidant capacity, and the concentration of high-density lipoproteins (HDL) in serum was conducted using enzyme-linked immunosorbent assay (ELISA) kits from SunLog. Specifically, the kits used were SL1949Hu_1 for PON1 enzyme activity, SL1999Hu_1 for total antioxidant capacity, and SL0878Hu for HDL concentration determination.

Statistical analysis

The statistical analysis IMB SPSS Statistics 26 program was used to detect the effect of different factors on study parameters. One – way ANOVA

and T-test were used to significantly compare between means. Chi-square test was used to significantly compare between percentage (0.05 and 0.01 probability). WINPEPI and SPSS program used to detect the genotyping.

Results and discussion

In the current study, we assessed a single nucleotide polymorphism within the PON1 gene in women who had experienced a minimum of two or three spontaneous abortions, in comparison to women without a history of recurrent spontaneous abortion (Table I). Table I related to information of different criteria obtained from the questionnaire forma answered by patients. According to the age results revealed no significant differences between controls and patients (29.46 ± 7.00 *versus* 29.78 ± 6.72, respectively). Also, no significant differences were noted between controls and RSA patients as related with body mass index (26.80 ± 3.62 *versus* 26.56 ± 3.84, respectively). The number of living child was in apparently healthy women significantly ($p< 0.05$) higher than that of women with recurrent spontaneous abortion (2.56 ± 0.91 *versus* 1.82 ± 1.89, respectively). No abortion in the apparently healthy women, while the mean number was 3.42 for the women with recurrent spontaneous abortion.

Table (1): Criteria obtained from questionnaire forma for apparently healthy subjects and recurrent spontaneous abortion patients in the present study.

Parameters	Apparently healthy subjects (Control)	Recurrent spontaneous abortion (Patients)	*P*- Value
Age (year)	29.46 ± 7.00	29.78 ± 6.72	0.8
Body mass index	26.80 ± 3.62	26.56 ± 3.84	0.7
Number of living child	2.56 ± 0.91	1.82 ± 1.89	0.01**
Number of abortion	o.o	3.42 ± 1.44	0.0001**

*** = Significant (P≤0.05).**
**** = Highly Significant (P≤0.01).**

In (Table 2) shows the PON1 activity, TAC and HDL concentration in serum of apparently healthy subjects and recurrent spontaneous abortion (RSA) patients. The activity of PON1 in serum of women with recurrent spontaneous abortion were significantly (P≤0.01) lower than apparently healthy women (8.15 ± 2.15 versus 9.66 ± 3.75 , respectively). In the same trend, the value of total antioxidant capacity in serum of women with recurrent spontaneous abortion were significantly (P≤0.01) lower than in apparently healthy women (2.80±0.10 versus 3.53±1.52, respectively).While, the serum concentration of HDL in women with recurrent spontaneous abortion were significantly (P≤0.01) higher than apparently healthy women (12.68±5.53 versus 9.01 ± 5.30, respectively).

Table (2): PON1 activity and HDL concentration in serum of apparently healthy subjects versus recurrent spontaneous abortion (RSA) patients. (Mean ± SD).

Serum parameters	Apparently healthy subjects (Control)	Recurrent spontaneous abortion (Patients)	*P*- Value
PON1 (IU/L)	9.66 ± 3.75	8.15 ± 2.15	0.01 **
TAC (U/ml)	3.53 ± 1.52	2.80 ± 0.10	0.008**
HDL (ng/ml)	9.01 ± 5.30	12.68 ± 5.53	0.003**

*** = Significant (P≤0.05).**
**** = Highly Significant (P≤0.01).**

In (Table 3) revealed the genotype and allele frequency of rs854560 SNP at PON1 gene in apparently the percentage of TA genotype was in women with recurrent spontaneous abortion significantly higher than that of apparently healthy women (60% versus 32 %, respectively, OR=3.5) and this represent a risk factor for recurrent spontaneous abortion incidence. Also, the percentage of AA genotype in women with recurrent spontaneous abortion was higher than that of apparently healthy women but non-significant (12% and 4% 68%, respectively). The values of allele frequency for T allele were 0.8 and 0.6 for apparently healthy women and women with recurrent spontaneous abortion, respectively. The values of allele frequency for A allele were 0.2 and 0.4 for apparently healthy women and women with recurrent spontaneous abortion, respectively. The RFLP analysis of PON1 polymorphisms revealed distinct electrophoretic patterns, with three different patterns being visually discerned on the gel. In particular, the genotype testing of the PON1 L55M polymorphism displayed the following results:

L: Ladder (50bp)

LL (TT) genotype (172 bp)

LM (TA) genotype (172, 103, 69bp)

MM (AA) genotype (103, 69bp) See (Figure1) for visual representation.

Table (3): Genotypes and alleles frequency of rs854560 SNP at PON1 gene in apparently healthy subjects versus recurrent spontaneous abortion (RSA) patients.

Rs 854560	Frequency, n (%)		X^2	OR (CI)
	Control	RSA patient		
Genotype frequency				
TT (LL)	32 (64%)	17 (34%)	--	1.00 (Reference)
TA (LM)	16 (32%)	30 (60%)	8.8697**	3.5 (1.5735 to 8.1405)
AA (MM)	2 (4%)	6 (12%)	3.1634	4.2 (0.7425 to 23.8770)
Allele frequency				
T (L)	0.8 (80)	0.6 (72)	--	1.00 (Reference)
A (M)	0.2 (20)	0.4 (44)	8.2813**	2.4 (1.3188 to 4.530)

RSA = recurrent spontaneous abortion; X 2 :chi square; OR: odd ratio; CI: confidence interval. N=50 for each group.

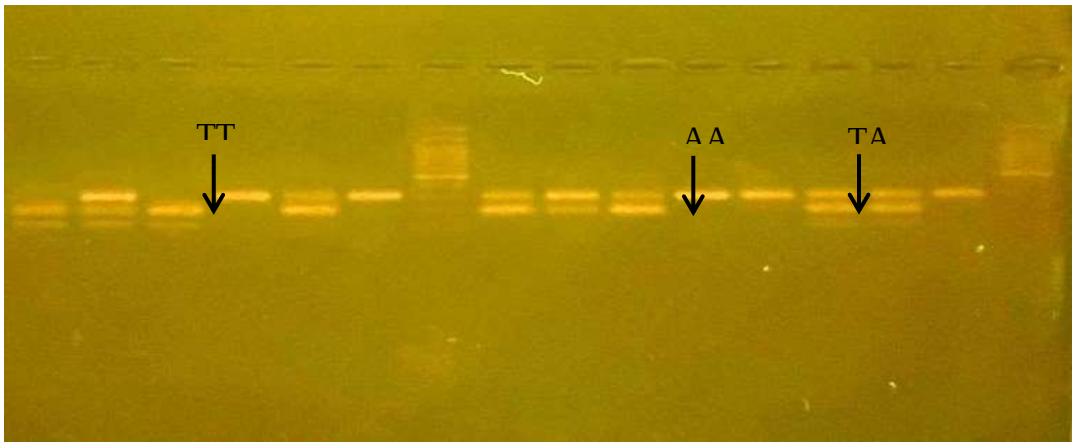

Figure (1): Gel Electrophoresis using RFLP analysis led to the detection of three different patterns observed on the gel (agarose 2.5%). Genotype testing of PON1 L55M polymorphism, 50bp ladder and 3 relative to the LL (AA) (172bp), LM (TA) (172, 103, 69bp), and MM (TT) (103, 69bp) genotypes, respectively.

The values of serum PON1 activity, TAC and HDL concentrations as related to recurrent spontaneous abortion and studied SNP are presented in (Table 4). Serum PON1 activity were unaffected by recurrent spontaneous abortion in this SNP. While, serum HDL concentration were in TT and TA genotypes significantly ($p<0.05$) higher than that in AA genotype .The total antioxidant capacity were unaffected by recurrent spontaneous abortion except with AA genotype was significantly lower in RSA than in apparently healthy subjects.

Table (4): Concentrations of PON-1 activity and HDL in serum of apparently healthy subjects versus recurrent spontaneous abortion (RSA) patients and as related to some SNPs. (Mean ± SD).

Rs 854560	Control	RSA	*P*- value
Serum PON-1 activity			
TT (LL)	11.66 ± 6.88	7.62 ± 2.39	0.4
TA (LM)	9.01 ± 3.89	8.60 ± 2.02	0.5
AA (MM)	6.14 ± 0.34	6.91 ± 0.56	0.2
***P*- value**	0.2	0.1	-
Serum HDL			
TT (LL)	9.60 ± 5.78	13.45 ± 5.69	0.002**
TA (LM)	8.75 ± 5.46	12.25 ± 5.76	0.07**
AA (MM)	8.58± 1.49	10.60 ± 3.13	0.5
***P*- value**	0.8	0.7	
Serum TAC			
TT (LL)	3.82 ± 1.93	3.04 ± 1.28	0.2
TA (LM)	3.04 ± 1.28	2.75 ± 0.83	0.1
AA (MM)	4.51 ± 0.53	2.29 ± 0.71	0.002**
***P*- value**	0.3	0.2	-

*** = Significant (P≤0.05).**
**** = Highly Significant (P≤0.01).**

Recurrent spontaneous abortion (RSA) is a frequent complication occurring during gestation, and it is a multifactorial condition. Previous research has indicated that the presence of specific alleles and genes in individuals can result in clinical manifestations, contributing to susceptibility to infertility issues, particularly RSA (11). One of the notable effects of these genetic factors is altered expression, which subsequently affects the function of enzymes involved in antioxidant roles. This alteration can either enhance or diminish the strength of antioxidants, disrupting the delicate balance between oxidative and antioxidant processes within the body, ultimately leading to the phenomenon known as oxidative stress. The PON1 enzyme is one of these enzymes that functions as an antioxidant. PON1 polymorphisms can affect this enzyme's activity, which in turn affects how effective an antioxidant it is. The body has several antioxidant defense mechanisms. The PON1 enzyme, one of these protective factors, is connected to high density lipoprotein and inhibits LDL oxidation, hence it might be suggested as a potential cause in avoiding RSA (9). According to that, when HDL concentration increase as PON1 activity decrease this lead to slowly oxidizing of LDL and increase the oxidative stress.

Free radical neutralization and oxidative stress control are the PON1 enzyme's primary and significant functions. Together with other enzymes and antioxidants, this enzyme fortifies the body's defenses against oxidizing chemicals. Previous research has shown that oxidative stress affects infertility

disorders like RSA, and since the PON1 is a well-known antioxidant, it may be essential in RSA inhibition (5). The PON1 enzyme's activity and, thus, its antioxidant capacity, can be increased or decreased depending on the type of polymorphism that is formed in this enzyme. Numerous studies suggest that oxidative stress has a part in infertility diseases, particularly RSA(12) . Total antioxidant capacity (TAC) testing is one of the best ways to assess oxidative stress. Evaluation of TAC offers insight into a person's antioxidant level in oxidative stress related disorders, such as RSA. Because oxidative stress causes the body's antioxidant capacity to be depleted, it has the potential to affect pregnancy (13).

Evidence suggests that different gene mutations affect PON's enzymatic activity in distinct ways. One of the PON1 polymorphisms that causes decreased paraoxonase activity is L55M rs854560, which results in methionine (M) being replaced with the amino acid leucine (L) at position 55. This polymorphism also includes a thymine to adenine mutation at nucleotide 163 in exon 3 (14,15). The serum enzyme concentration varies as a result of the rs854560 polymorphism. AA genotypes had a higher PON1 enzyme content than TT genotypes (16). The rs854560 (L55M) polymorphism is linked to a higher risk of heart disease, cancer, and stroke (17,18).

The results of this study showed that the heterozygous TA genotype is related with the risk of RSA and the minor allele (A allele) increased in women with RSA. These findings contrast with the results from a study conducted on the Iranian population, which indicated a significant association between the L55M polymorphism's MM (AA) genotype [OR = 3.593, CI = 0.972–13.280, p = 0.042]. In this Iranian study, a meaningful relationship was observed between the control and case groups concerning the MM genotype, suggesting that individuals with the AA genotype face an elevated risk of recurrent spontaneous abortion (RSA) (10). Another study suggested that individuals with the AA genotype might be more susceptible to infertility, whereas those with the heterozygous (TA) genotype could potentially exhibit some level of protection against infertility. (19). In a prior study, it was established that individuals with the mutant MM genotype and the heterozygous LM genotype had a nearly fourfold higher likelihood of experiencing miscarriage compared to those with the wild-type LL genotype (20). The study was to explore the relationship between PON1 polymorphisms and Recurrent Pregnancy Loss (RPL) by assessing the impact of oxidative stress induced by various enzyme polymorphisms. To achieve this objective, we referred to multiple studies that had investigated the influence of oxidative stress on infertility have been conducted.

Conclusion

This study revealed the correlation between recurrent spontaneous abortion in sample of Iraqi women as it occur by increasing the oxidative stress in the body by decreasing the activity of PON1 antioxidant enzyme and TAC affected by L55M polymorphism and it is a risk factor for RSA.

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