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Polymorphism of MnSOD (Val16Ala) Gene in Blighted Ovum

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ABSTRACT

Blighted Ovum or anembryonic pregnancy (empty pregnancy) is the most common reason of abortion in first three months of pregnancy. Manganese Superoxide Dismutase (MnSOD) is among the most important anti-oxidants of human immune system. It is located on 6q25 chromosome but, acts on mitochondrial matrix. About 90% of produced ROS in human cells are removed by MnSOD. In case of mutation or inactivity of this enzyme, mitochondrial and nuclear DNA will severely be destructed. The most common polymorphism of its gene is Val16Ala. The purpose of current study was investigating a possible mutation in women who had to abort during first two months of pregnancy because of blighted ovum. In a case-control study, 34 patients and 34 healthy subjects were entered. Genome DNA was sampled from saliva and its genotype was determined using Tetra Primer ARMS-PCR technique. Statistical analyses were carried out by Madcalc (Version 12/1) software. According to the results, respective frequency of TT, CT and CC genotypes were 48%, 50% and 2% in patients and 22%, 17% and 61% in control group. Statistical analysis revealed a significant relationship between Val16Ala polymorphism of MnSOD gene and Blighted ovum (P = 0.0001). Moreover, there was significant relationship between allelic frequency in patients (C=27 and T=73) and control group (C=71 and T=29). (P=0.003, 95% CI = 0.0018, OR = 0.0168). Based on the results obtained from our experiments, it is concluded that a significant relationship exists between Val16Ala polymorphism of MnSOD gene and blighted ovum.

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1- Introduction

Blighted ovum is one of the most common reasons of abortion during first three months of pregnancy (Coughlin and Roberts, 2004). After fertilization of ovum with sperm, the fertilized ovum will naturally have planted in womb and the cell divisions commence (Wang et al., 2003). Continual cell division will form placenta and pregnancy sac but embryonic division may stop during an uncommon progress while pregnancy sac had formed. This situation is called anembryonic pregnancy or Blighted Ovum (Coughlin and Roberts, 2004). Its reason is completely unknown but, researchers believe that genetic and chromosome disorder is the probable cause of this disease (Wyatt et al., 2005). Anembryonic pregnancy is the main reason of about 50% of abortions during first three months of pregnancy. Missed or irregular periods, mild abdominal pains, pain and swelling in breasts, brown vaginal discharge, positive pregnancy test and supposal of normal pregnancy are main symptoms of this disorder. Although no embryo had formed but placenta continue to growth and

pregnancy hormones will secrete from placenta to mother's blood (Faghihzadeh et al., 2003). Anembryonic pregnancy or Blighted Ovum may be diagnosed during sonography at the end second month which indicates pregnancy sac with >20 mm diagonal while no embryo exists. The most common reason may be chromosome disorder in fertilized ovum. Increased or decreased number of chromosomes may suspend cell division of primary zygote. Low quality of ovum or sperm is the other reasons that may cause blighted ovum. According to reports, blighted ovum is the main reason of one-third of abortions before eighth weeks of pregnancy (Shekoohi et al., 2013). Mitochondrion is one of the most important places in cell for aggregation of reactive oxygen species (ROS) and free radicals (Von et al.,1990). It is noteworthy that aerobic organisms with cellular respiration require respiratory chain in inner membrane of mitochondrion. Free radicals such as superoxide (O^{-2}) are among by-products of cellular respiration. Presence of MnSOD in mitochondrial matrix is a defensive mechanism of cells which remove radicals and poisonous materials by acting as a defensive antioxidant (Lin et al., 2003). This is a homotetramer metalloprotein with four units of manganese in every subunit. These manganese molecules are co-factors which facilitate catalytic process of enzyme reaction (Lin et al., 2003). Expressing gene of this enzyme with 83466 base pairs is located on long arm of 6q25 chromosome and includes 5 exons and 4 introns (Honda and Honda , 1999). The gene includes various polymorphisms in coding and promoter regions and C/T polymorphism in Val16Ala coding region is one of the most commons. Computer analyses, test results and laboratory errors have revealed that A variant (Alanine) will create an alpha helix structure of MnSOD which may result in excessive transportation of enzyme from mitochondrion membrane, while V variant (Valine) will create beta sheet structure which may result in decrease (30 to 40 percent) of enzymes passage from mitochondrial membrane (Mitrunen et al, 2001). Therefore, MnSOD enzyme with valine is facing for ROSs. The result is endangering cell and DNA (Ambrosone et al., 1999).

SOD (EC 1.15.1.1) is an important antioxidant enzyme in cell catalyzing break down of superoxide anions to hydrogen peroxide and molecular oxygen. SODs are categorized in three different classes on the basis of metal cofactor in their structure:

1. SOD1 or Cu/Zn SOD: in form of soluble dimer in cytoplasm of eukaryote cells and requires copper and zinc for its catalyzing activity.
2. SOD2 or MnSOD: by homotetrameric forms in mitochondrial matrix of eukaryote cells and one manganese ion is located in its every subunit.
3. SOD3 or extracellular SOD which is discovered lately and includes copper and zinc (Zelko et al., 2002).

As most women tolerate severe stresses during pregnancy, providing stable conditions for them is highly required. Accordingly, it is necessary to investigate the extent of antioxidants in their body's fluids and activity of genes which produce these antioxidants. In this study, polymorphism changes of MnSOD gene were investigated in women with blighted ovum. Due to its role as one of the most efficient antioxidants in human cells and significant activity changes it undergoes during ovarian cycle and pregnancy, MnSOD was selected for our present work.

2- Methodology

In a case-control study, 34 women with blighted ovum as test group and 34 healthy women as control group were selected. The aim of research was explained for them and informed consent was obtained from each case. After a rinse with distilled water, 5 ml of both group's saliva was sampled in Falcon sterile tubes and stored in -20 ^{0}C for later experiments. In next stage, DNA was extracted by GPP solution (Sinaclon Company) and identified through spectroscopy and placing DNA on gel electrophoresis. DNA was then replicated through allele-specific PCR tetra primer (ARMS-PCR) method and probable Val16Ala polymorphism in MnSOD in these four replicated primer was investigated by Oligo 7 primer analysis software. Primers employed for the alleles were as follows:

C: (5 CGGTAGCACCAGCACTAGCA3) Fc and (5 TGGAGCCCAGATACCCCAAAG3) Rc

T: (5 CCACTCAAGTACGGCAGAC3) Ft and (5 TGGAGCCCAGATACCCCAAAA3) Rt

Taq enzyme, $MgCl_2$ and dNTP buffer, water and DNA were employed to replicate the required gene. The temperature cycle for PCR was as follows: denaturation temperature 94°C (5 min), annealing 59°C (45 seconds) and sent ethic temperature of 72°C (5 min) for 35 cycles. The PCR product for T-allele was a band with 688 base pairs and a band with 350 base pairs for C-allele. According to the results, all case and control samples were replicated and the PCR process was performed (once) for T-allele and C-allele. Each sample was then analyzed on the basis of provided

350 and 688 base pairs bands. Samples with one 688 base pairs band belonged to healthy subject with TT genotype. Samples with both 350 and 688 base pairs bands are CT heterozygote and the ones with one 350 base pairs band have CC mutated homozygote. (Figure 1)

3- Results

The study was performed as a case-control trial by sampling from 34 women with blighted ovum as case group and 34 healthy women as control group. The ages of both groups were between 20 to 35 years old. After sampling their saliva, ARMS-PCR method was used for DNA replication and Madcalc (version 12/1) software was employed for statistical analysis. The PCR products are presented in Figure 1.

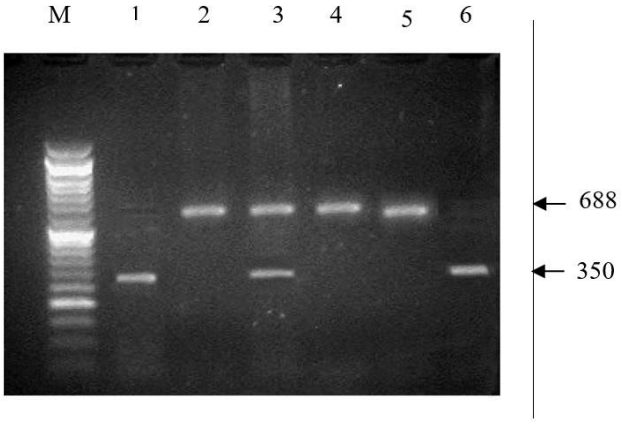

Figure 1: PCR products of T and C Alleles. Person with TT genotype: 1, 4, 5. person with CT genotype: 3 and person with CC genotype: 1, 6

Among 34 samples with Blighted Ovum, 7 (20%) cases had Val/Val genotype, 6 (17%) were heterozygote and had Val/Ala genotype and 21 (61%) had Ala/Ala genotype. Among control group, 16 (47%) items had Val/Val genotype, 17 (50%) had Val/Ala genotype and 1 (0.02%) had Ala/Ala genotype. Genotype frequency among case and control group was investigated through Chi-square test (P-Value = 0.0001). Therefore, there was significant difference between case and control groups ($P < 0.05$). Considering significant of *P*-value, it was required to calculate OR and CI. Odd Ratio (OR) was calculated by Madcalc software too. The results are as follows: (OR = 0.0168, 95% CI = 0.0018 – 0.153, P = 0.0003) (Table 1). Then the frequency of T- allele and C-allele was calculated in both case and control groups. Respective frequency of T and C alleles in case group was 73% and 27% while, this value in control group was equal to 29% and 71% (Figures 2, 3) and Table1.

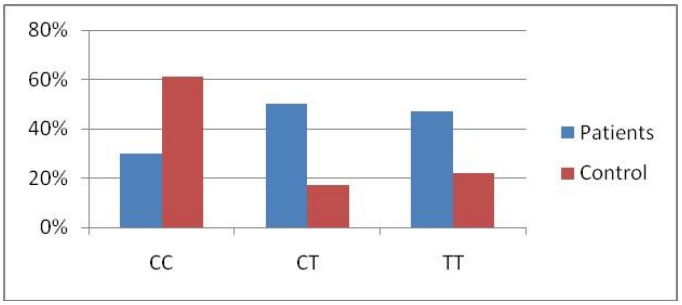

Figure 2: Bar diagram of MnSOD genotypes. Significant difference is observed between CC, CT and TT genotypes of case and control groups.

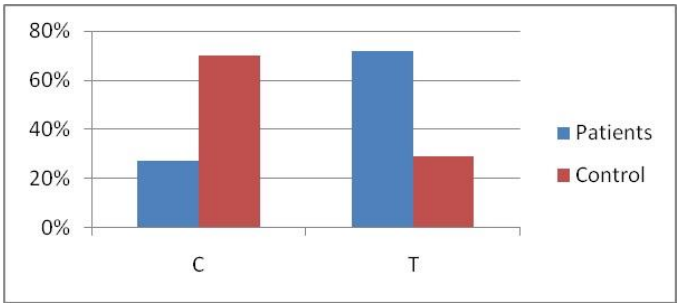

Figure 3: Frequency of C and T alleles in MnSOD. As indicated above, significant differences exist between case and control group.

In summary, statistical analyses on the studied population revealed that MnSOD polymorphism shall be considered as a risk factor in women with Blighted Ovum. However, we suggest further studies with larger and wider population.

4- Discussions

The results of current study which investigated importance of Val16Ala polymorphism of MnSOD in Blighted Ovum disease revealed the important role of antioxidant enzymes in human immune system and the relationship between changes in gene expression of these enzymes with various diseases including Blighted Ovum. In a study on women with Blighted Ovum it has been shown that echo sound during sonography in patients is different from women with normal pregnancy (Bernard and Cooperberg, 198). In women with Blighted Ovum, the sounds are weaker because of existence of empty pregnancy sac with approximate size of 2 cm (Bernard and Cooperberg, 1985). The size of pregnancy sac is one of the most important factors which lead physicians in diagnosing probable disease in pregnant women. Size of pregnancy sac in women with Blighted Ovum is about

Table 1: Frequency of alleles and genotypes of MnSOD in patients and control groups

	Control N (Frequency %)	Patients N (Frequency %)	P-Value
Genotype	-	-	P = 0.0003
CC	21 (61%)	1 (2%)	
CT	6 (17%)	17 (50%)	OR = 0.016
TT	7 (22%)	16 (48%)	
Allele	-	-	P = 0.0002
T	29%	73%	-
C	71%	27%	-

1.8cm while, in normal cases it is 1.3cm (Bernard and Cooperberg, 1985). They reported that 46% of investigated pregnancies were unsuccessful because of various reasons but, only one-fifth of them that had clinically diagnosed and resulted in spontaneous abortion. Blighted Ovum is a reason of considerable percentage of unsuccessful pregnancies which main cause is presence of an abnormal Karyotype (Shekoohi et al., 2013). In 1978, a study was performed on 1500 women with Blighted Ovum who had to undergo abortion. 61% of this population had abnormal Karyotype and the most common disorders in them were as follows: 52% Autosomal Trisomy, 20% Triploid, and 28% Monosomy X (Shekoohi et al., 2013). In a normal cell, there is a balance between ROS value and antioxidant defense. Therefore, MnSOD can convert peroxide free radicals to H_2O_2 and O_2. Then the H_2O_2 will be discomposed by GPX1 and Catalase and changed to water. High value of MnSOD (in Ala form) may disarrange such balance. Accordingly, H_2O_2 will be continually produced but, there are not enough GPX1 and Catalase to remove it (Van Remmen et al., 2003). The result is an imbalance between these three enzymes and concentration of H_2O_2 in cell which endanger DNA (Collins and Duthie, 1995). Concentration of H_2O_2 in cells is highly related to increase of Tumor Necrosis and Apoptosis in cells and may increase cell proliferation rate by creating a protein kinase pathway. Importance of MnSOD enzyme resulted in investigation of its role in different diseases including breast cancer, prostate cancer, gastric cancer, rectal and colorectal cancer, lung cancer, kidney cancer, many neurodegenerative diseases including Alzheimer and Parkinson, aging process, infertility and spontaneous abortion and also in studying mental and behavioral disorders (Mitrunen et al., 2001; Van Remmen et al., 2003]. Therefore, further investigations to clearly explore the role of this important antioxidant enzyme in unwanted abortions are highly recommended when considering the higher risks of oxidative stress during pregnancy.

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