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ASSOCIATION OF GLUTATHIONE S-TRANSFERASE M1 AND T1 POLYMORPHISMS WITH SEVERE OLIGOZOOSPERMIA

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ABSTRACT

The aim of this study was to examine whether an association exists between glutathione S-transferase M1 (GSTM1) and glutathione S-transferase T1 (GSTT1) genes polymorphism and severe oligozoospermia. Blood samples from patients and healthy individuals were collected and used for isolation of genomic deoxyribonucleic acid (DNA). The polymorphisms were analyzed using multiplex-polymerase chain reaction (multiplex-PCR) technique. Data were analyzed using Chi-square, Fisher exact test and independent t-test, as appropriate. The frequency of GSTT1 and GSTM1 null genotypes was observed to be higher in infertile men with severe oligozoospermia 41.18% and 27.45% in comparison with 13.46% and 9.62% in the fertile men, respectively.

Keywords: Glutathione S transferase, GSTT1, GSTM1, severe oligozoospermia, Male factors, infertility, Polymorphisms

INTRODUCTION

Infertility is a major problem worldwide, about 10 to 15% of couples of reproductive health has been affected by at least 50% of their male factor infertility as the most important factor leading to the decline and disorganization semen quality are (Boivin *et al.,* 2007; Safarinejad, 2008). Although there are many factors to damage male fertility, but in about 30% of them, are still the main reason for infertility is not known and hence this type of failure is known as idiopathic infertility (Pasqualotto *et al.,* 2006). However, researchers believe that damage to spermatozoa genetic composition plays a vital role in male fertility as far as say, any defects in these compounds can be among the leading causes of infertility in men. Studies show that certain genetic polymorphisms are associated with damage to spermatogenesis in male infertility and can lead such as azoospermia oligoospermia unknown (O'Flynn *et al.,* 2010). According to the criteria of the World Health Organization (WHO) sperm count below 20 million per mL (Oligozoospermia) motility less than 50% (Asthenozoospermia) and morphology (Morphology) less than 30% (Teratozoospermia) in semen is abnormal and may cause infertility. Severe Oligozoospermia are examples in which the number of sperm per mL of semen is less than 5 million (Rowe *et al.,* 2000)]. Because of the high unsaturated fatty acids in the sperm plasma membrane and the lack of protective enzymes in the cytoplasm is particularly vulnerable to oxidative stress. ROS production of spermatozoa in their semen origin first and then several nuclear leukocytes (Hammadeh *et al.,* 2006). The body is defenseless against oxidative stress, glutathione S-transferase (GST) as the most important antioxidant defense system against oxidative stress is known to be able to decrease reactive oxygen species metabolic by substances with less (Dusinska *et al.,* 2001), and DNA damage caused by lipid peroxides, easily resolve domestic product (Hurst *et al.,* 1998). Therefore, any change in the genes encoding glutathione S-transferase, which leads to a reduction or loss of enzyme activity, it can be important for the assessment of oxidative stress infertility. Any increase in the level of reactive molecules called oxygen species (ROS) can be seen as an important factor in male infertility (Saleh and Agarwal, 2002), because these species are capable of oxidative stress and damage to DNA strands and breaking it , affect sperm and cause infertility or at least low fertility provide this. Glutathione S-transferase phase II antioxidant enzymes belong to a large family in a variety of physiological intracellular detoxification participate (Ketterer, 2001). Nowadays knowledge GST in 8 categories including: alpha, mu, kappa, omega, pi, Sigma, Theta and Zeta, which is the basis of the difference in the amino acid sequence of the 8 groups, respectively, by gene by GSTA, GSTM, GSTK, GSTO, GSTP, GSTS, GSTT and GSTZ coded (2Safarinejad *et al.,* 2012). In addition, each group has several genes and isoenzymes as well (Hayes and Strange, 2000). Glutathione-S-transferase a transporter in conjunction biological variety of endogenous and exogenous compounds such as toxins (poisons) or carcinogens (cancer-causing) to their metabolism is associated with reduced glutathione (Hayes *et al.,* 2005; Strange *et al.,* 2008). Any reduction in the GST from the reaction of electrophilic, can effect on the micro molecule cell, such as nucleic acids, lipid and protein, affect spermatogenesis and their activity (Wu *et al.,* 2008). In humans, the GSTM1 gene into a gene cluster of genes GSTM1-GSTM5 on chromosome 1P13.3 is located and has a variety of custom functions and is important,

people with homozygous deletion of the status of this gene lacks enzymatic activity of cytosolic GST-μ (Pearson *et al.,* 1993). Many reports indicated that this type of relationship with an increase risk of infertility in men is homozygous (Safarinejad *et al.,* 2012). In one study, GSTM1 gene polymorphisms with male infertility varicocele was examined and removed this gene in patients with varicocele causes performance degradation resulting in decreased antioxidant capacity of sperm and semen in these patients because of the null genotype GSTM1 (Chen *et al.,* 2002), also showed the study of Aydemir that the genotype GSTM1, increased oxidative damage spermatozoa in the semen of men with infertility is unknown (Aydemir *et al.,* 2007). Polymorphisms of GSTM1 in patients may be an important resource for spermatozoa to oxidative damage, and DNA fragmentation of sperm tend to be followed (Rubes *et al.,* 2007). On the other hand the ta group human GST enzymes (GSTT) of two subunits, GSTT1 and GSTT2 which are both located on chromosome 22q11 (Tan *et al.,* 1995). GSTT1 gene polymorphism in position from the elimination of the gene in people with genotype can also be empty, the virtual absence of enzyme activity cause (Pemble *et al.,* 1994). Previous studies, GSTT1 null genotype of a possible link between person's susceptibility to diseases such as cancer, diabetes and cardiovascular failure point, but the association of these genotypes with significantly less male infertility (Wu *et al.,* 2008). Wu did a study on men northwestern China, results showed provide GSTT1 null genotype that sometimes the groundwork for a failure such as azoospermia and oligozoospermia unknown (Wu *et al.,* 2008). In another study of infertile men with varicocele China showed that oxidative stress can cause infertility in patients with GSTT1 null genotype risk varicocele and oxidative stress in patients with varicocele increases to some extent (Wu *et al.,* 2009). However, extensive studies, the researchers showed that these genotypes (genotypes null) in the genes GSTM1 and GSTT1 with unexplained infertility in men there and coordination (Song *et al.,* 2014). However, to support this important and help to preserve fertility in these patients, further research is necessary, this necessity prompted us to offer this study, we focused on the analysis of this matter.

MATERIALS AND METHODS

In this study, 51 infertile men with severe oligozoospermia and 52 healthy men aged 20–37 years were selected randomly in 2014. All of the selected patients were severe oligozoospermia with normal 46XY karyotype and had a positive history of male factor infertility.Inclusion criteria were 20-35 year-old men with azoospermia and exclusion criteria was azoospermic men with not severe oligozoospermia or any chromosomal abnormality.

Study of infertile men with severe oligozoospermia including patient history, family history, semen analysis and laboratory tests, including hormonal profile and karyotyping. Various factors such as age, blood group and occupation extracted from the patient and then both case and control groups in terms of polymorphisms in genes GSTT1 and GSTM1 were examined.

Genomic DNA was extracted from peripheral blood of all patients and controls. DNA was extracted by salting out from all blood samples.Quality and quantity of extracted DNA were performed using spectrophotometry and electrophoresis agarose gel respectively.

Three different primer pairs for the detection of genes GSTT1 and GSTM1 homozygous deletion (Null genotype)were used by multiplex PCR.Three pairs of primers including GSTT1, GSTM1 and B-Globin(as internal control) used in this study.Primer pairs were studied using the bioinformatics softwares Allele ID7, Primer premier 5 and Base stacking Tm Online. Fragments of GSTT1 (480 bp), GSTM1 (215 bp), and B-globin (268 bp) genes, the last one used as an internal amplification control to exclude false negatives, were simultaneously amplified.

GSTT1:
Forward (F): 5′- TTC CTT ACT GGT CCT CAC ATC TC -3′
Reverse (R): 5′- TCA CCG GAT CAT GGC CAG CA -3′
GSTM1:
F: 5′- GAA CTC CCT GAA AAG CTA AAG C -3′
R: 5′- GTT GGG CTC AAA TAT ACG GTG G -3′
B-globin:
F: 5′- CAA CTT CAT CCA CGT TCA CC -3′
R: 5′- GAA GAG CCA AGG ACA GGT AC -3′

DNA amplified by multiplex PCR method. PCR amplification condition had a thermocycling procedure consisted of 4min in 94^{o}C for initial denaturation. The procedure followed by 32 cycles of 30s at 94^{o}C, 30s at 59^{o}C and 4min in 65^{o}C with a final extension at 65^{o}C for 5min.

Gel electrophoresis

The products of PCR were run by electrophoresis on a 1.5% agarose gel.

Statistical analysis

X^2 test,t-test and fisher's exact test were carried out to compare difference between the cases and controls. The statistical analyses were performed with SPSS 16 statistical software when p-value was under 0.05 the difference was considered significant.

RESULTS

In this study, 51 patients with severe oligozoospermia with an average age of 20-35 and 52 healthy men with an average age of 20-37 were studied. All patients and controls gave written informed consent.

The length of amplicons was 215bp for GST-M1, 480 bp for GST-T1, and 268 bp for β-globin. Negative examples, GSTM1 and GSTT1 genes lack either separately or together in the presence of B-Globin gene null genotype for each is indicated. In positive samples of each gene separately or together in the presence of B-Globin gene expressed as the wild genotype. In Fig. 1, L represents a Ladder or size markers, the first column is a positive control, mode (Poongothal *et al.*, 2009) is a wild type genotype, mode (Boivin *et al.*, 2007) is a GSTT1 null genotype, mode (Safarinejad, 2008) is a GSTM1 null genotype, mode (Pasqualotto, 2006) is GSTT1 and GSTM1 null genotype and last column is negative control.

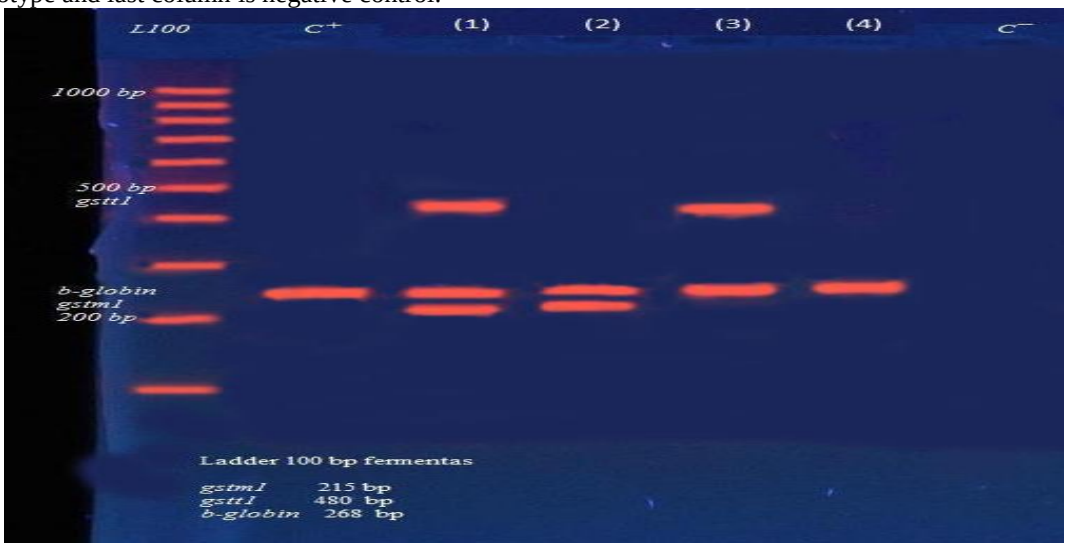

Fig. 1.. Multiplex PCR B-globin genes and GSTM1 and GSTT1

The GSTM1 and GSTT1 gene deletion in the patient group (severe oligozoospermia) and controls (healthy) were different, and this difference by chi-square test for GSTM1 gene level ($P < 0.05$) and GSTT1 gene level ($P < 0.01$) were significant. The deletion of the GSTM1 gene in two groups of patients and control subjects was 27.45 and 9.62 , respectively. While eliminating the GSTT1 gene in two groups of patients and control subjects was 41.18 and 13.46 respectively. The values shown in the Table 1.

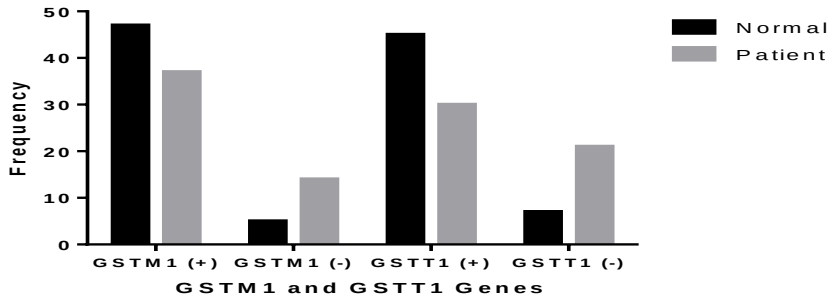

Fig. 2. GSTM1 and GSTT1 genes in the presence and absence of control groups and patients.

Table 1. Percentage of GSTT1 and GSTM1 genes in the presence and absence of controls and patients.

P- value	Number of control	Number of patient	Genotype	Gene
	5 (9.62%)	14 (27.45%)	Null	
0.02				GSTM1
	47 (90.38%)	37 (72.55%)	Presence	
	7 (13.46%)	21 (41.18%)	Null	
0.002				GSTT1
	45 (86.54%	30 (58.82%)	Presence	
	0 (0%)	5 (9.80%)	(Either both null)	
0.0004				GSTM1 and GSTTT1
	12 (23.08%)	25 (49.02%)	Either one null	
	40 (76,92%)	41 (78.21%)	(Both present)	

According to Fisher's exact test the strength of the relationship between GSTT1 and GSTM1 genes and oligospermy in patients either were the relative risk (RR) or the odds ratio (OR) and the results show the risk of the disease by removing the gene M1, 95% and Gene T1, 99% . Fisher's exact test was used for both genes with less than 0.05 and 0.01, P-Value for GSTM1 and GSTT1 genes respectively. Resulted data showed that there is a significant relationship between the absence or presence of GSTT1 and GSTM1 genes and oligospermy. This means that the removal of these genes for patients is significantly higher than in healthy individuals. The relationship between genes and oligospermy (R R) also was conducted. The amount of the GSTM1 and GSTT1 genes are 2.1 and 2.4 respectively. The estimated odds ratio test (OR) for both genes, GSTM1 and GSTT1 genes, in which the amount equal to 3.5 and 4.5, respectively, were calculated. These values also show a significant association of genes with oligospermy. This means that the absence of these genes is a risk for oligospermy. (Table 2).

Table 2. The relationship between the combined GSTM1 and GSTT1 genotypes and the risk of severe oligozoospermia.

M1 & T1 Combined	Control	Cases (Oligo Spermi)	OR(95 %CI)
Both present	40	21	1(reference)
Either one null	12	25	3/968 **(1/666 to 9/451)**

T-test was performed for each gene separately. By using of this test also a significant difference between patients and controls were observed. As for the differences in the GSTM1 gene ($P < 0.05$) for GSTT1 gene at the level of $P < 0.01$ were significant. The frequency of the deletion of these two genes in the studied population of healthy and patients was found significant differences. Although each of these genes separately, significantly reduces the possibility of oligospermy. Although both genes simultaneously also significantly reduces the likelihood of oligospermy. It can be said that a significant relationship between presence of these genes and the oligospermy are removed. Of course, this needs further studies.

DISCUSSION

Because in many cases the cause of infertility can be the continuation of a joint life and peace of mind for families, male infertility could be treated in the overall planning. Many different genes in the germ cells, somatic cells in the testes, especially the Sertoli cells epididymis genital cutting and other states are crucial to the full development of sperm function, in other words, the destruction of many paths and different cellular networks may lead to male infertility (Liska, 2003). studies have shown Glutathione S-transferase active that sperm levels (Gopalakrishnan *et al.,* 1998). Human cytosolic GST genes can show genetic polymorphisms that instructs specialized construction crews defense capacity against oxidative stress enzymes affected and advancing the failure to provide some of this time can be an increased risk of male infertility include (Ryberg *et al.,* 1997). In the present study, GSTT1 and GSTM1 genotype frequency genes in men with severe oligozoospermia sequence 41.18% and 27.45% in the control group by 13.46% and9.62%, respectively. Frequency of GSTT1, GSTM1

genes between the two groups is significant. Previous studies of glutathione S-transferase polymorphisms on male infertility had mixed results. Most of these studies of polymorphisms of GSTM1 gene, GSTM1 and sexual hormones including estradiol and testosterone connection feature because it is a protein necessary in the course of spermatogenesis and antioxidant enzyme activity of this gene also leads to more considered. Wu and colleagues in a study that was conducted on 6934 patients, demonstrated between polymorphisms of GSTM1 and GSTT1 null and there is an increased risk of male infertility (Wu *et al.,* 2013). Chen in his study showed that sperm of patients with varicocele and null genotype of GSTM1 is more vulnerable to oxidative stress (Chen *et al.,* 2001). Some differences in results may be due to the difference in the race of the subjects and the effect of polymorphisms of this gene family.

CONCLUSION

It can be concluded that the absence of these genes significantly decreased glutathione S-transferase antioxidant activity. In this situation the body's defense against stress, free radicals and oxidants decreased and therefore the situation can be oligozoospermia. This decrease sperm count can be caused by damage to sperm DNA due to imbalance between antioxidants and free radicals and oxidants. Finally, we can say that there is a significant correlation between these two genes null genotypes and the oligozoospermia , of course, this requires further studies.

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