Association of glutathione S-transferase (GSTT1 and GSTM1) polymorphism with varicocele: an Iranian case-control study

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Key words: Varicocele, GSTT1, GSTM1, polymorphism, sperm parameters.

http://dx.doi.org/10.12692/ijb/4.5.146-153 Article published on March 10, 2014

Abstract

Glutathione S-Transferase (GST) gene family have subclasses such as GSTM and GSTT that present on the human sperm surface and play an important role against oxidative stress. In this case-control study, the polymorphisms of GSTT1 and GSTM1 in association with sperm parameters were studied which were involved 46 men with varicocele and 48 men without varicocele as controls. Semen analyses were carried out according to WHO guidelines and Blood DNA was extracted using salting out procedures. GSTT1 and GSTM1 polymorphism gene determined through multiplex-PCR respectively. Frequencies of GSTM1 null genotype in cases and control groups were 60.9 and 41.7, respectively. There were no statistically significant differences between GSTM1 null and positive genotype in two groups (p>0.05). Frequencies of GSTT1 null genotype in the case and control groups were 47.8 and 50, respectively. There were no statistically significant between GSTT1 null and positive genotype in two groups (p>0.05). Deficiency of enzyme activity in GSTM1 null genotype were not affected on morphology, slow and quick progressive of sperm, but caused the statistically decrease in count of sperm between GSTM1 null and positive genotype. In the case of GSTT1, the effect of GSTT1 null genotype, no affected on sperm parameters that compensate activity of this super family gene may justify the cause.

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Introduction

Infertility is defined as the inability of a couple to conceive after 12 months of regular, unprotected intercourse. About 10%-15% of couples suffer from this problem (Keye et al., 1995). Based on reports in about 40% of infertilities male factor is involved in an extent. Generally, male infertility is due to defects in production or sperm development process in the testis or male reproductive tracts and divided into pre testicular, testicular and post testicular causes (Jafari, 1995).

Most of infertile men have a deficiency in spermatogenesis. Some of the most important causes of this type of infertility are chromosomal abnormalities, chemical agents and chemotherapy, radiation, occupational gonadotoxins, general diseases and exposure of the testis to increased temperature. Some diseases like cryptorchidism, testicular inflammation and varicocele have an important role in infertility (Jafari, 1995).

A varicocele is a widening of the veins along the cord that holds up a man’s testicles (spermatic cord). About 10%-15% of young men and 30% of elderly men have this problem (Sandlow, 2004, Walsh et al., 1998). The exact cause of developing this disease is not determined yet, but some hypothesizes are proposed like hereditary causes, nut cracker phenomenon, congenital absence of one way bicuspid valves of testis veins and increased pressure due to a long and vertical path of the left testicular vein (Jafari, 1995). Varicocele can disturb the production and function of sperm (Walsh et al., 1998, Lipshultz and Corrier, 1977) but the exact mechanism is not known yet. Some proposed hypothesizes are hypothalamus-gonadal pathway disturbance, testicular hypoxia due to venous stasis, increased testis temperature, reflux of toxic metabolites from kidney or adrenal and increased oxygen free radicals (Diamond et al., 2004). Recent studies show that testis function disturbance due to oxidative stress is involved in many of mechanism that cause infertility in varicocele (Naughton et al., 2001).

All living organisms need oxygen for survival. Major reactive oxygen species (ROS) are O$_2^-$, OH and H$_2$O$_2$ that can invade to biomolecules and cause harmful changes in cellular structure (Turner and Lysiak, 2008). There are several reports on increased super oxide anion in semen and testicular tissue of patients with varicocele (Burnbaugh et al., 2007, Allamaneni et al., 2004). ROS production is a physiologic process and has an important mediatory role in signal transfer, sperm capacitation, acrosomal reaction facilitation and oocyte-sperm fusion (Cam et al., 2004). But due to high concentration of unsaturated fatty acid in plasma membrane and low level of sweeper enzymes in cytoplasm of sperm, it is highly sensitive to non physiologic oxidative stress (Saleh and Agrawal, 2002). Lipid peroxidation of unsaturated fatty acid at the head and middle part of sperm cause decreased motility and dysfunction in oocyte-sperm fusion reaction (De Lamirande and Gagnon, 1995).

Varicocele can cause disturbance of total antioxidant capacity (TAC) of semen plasma (Alvarez et al., 1987, Smith et al., 1996). Then body must decrease ROS to prevent sperm damage and fertility decrease (Chen et al., 2001, Alkan et al., 1997).

Glutathione-S-transferase (GST) enzyme family is a defense mechanism against oxidative stress damages. This enzyme family includes a large number of phase II enzymes that work in the detoxification of xenobiotics (Alkan et al., 1997). Presence of Glutathione-S-transferase (GST) in sperm has been proved by several studies (Agarwal et al., 2006). Contrasting with somatic cells, because of high levels of unsaturated fatty acids, especially at the head of the sperm, it is more sensitive to reactive oxygen species (ROS). Then, if the function of Glutathione-S-transferase is disturbed in sperm has been exposed to oxidative stress, the plasma membrane of sperm will be damaged and then will cause decreased ability of sperm to fertilize the oocyte at in vitro environment (Agarwal et al., 2006, Armstrong, 1997).

GST gene divided into six subgroups of α, µ, π, δ, θ
and ξ. Although some locus polymorphisms are detected in all six classes of GST family genes, but majority of them are on GSTM1 and GSTT1 that are located on chromosomes 1 and 22 respectively. GSTM genes are a cluster of genes consisting GSTM1 and GSTM5 located at 1P13*3 (Gopalakrishnan and Shaha, 1998). Any change in GST gene coders can cause decrease or absence of enzyme function. Considering with the key role of GST in defending against oxidative stress and its role in oocyte-sperm fecundation, absence of decrease in this enzyme can cause male fertility disorder (Wu et al., 2009). The aim of this study was to determine GSTT1 and GSTM1 polymorphism in healthy and patients with varicocele and its relation to sperm parameters.

Materials and methods

Study Design

After institutional ethics committee approval, this case control study was done in Research and Clinical Center for Female Infertility, Yazd, Iran. Ninety four participants (46 confirmed varicocele men as a case group and 48 confirmed healthy men in the control group) were included in the study for detecting GSTT1 and GSTM1 gene polymorphism. Patients were allocated according to age and weight. For all patients sperm analysis based on World Health Organization (WHO) was done. The parameters of sperm analysis were sperm count, morphology and motility (fast and slow).

Genomic DNA Extraction

Five ml peripheral blood was taken from participants and maintained under -20°C in tubes containing EDTA. DNA extraction was done by salting out method and the Quantity and the quality of extracted DNA were assessed by spectrophotometry and agarose gel methods.

Polymerase Chain Reaction (Multiplex PCR)

To determine homozygote deletion in GSTT1 and GSTM1 by Multiplex PCR method, three pairs of GSTM1, one pair of GSTT1 and one pair of Beta-Globin primers were used for confirmation of amplification. For the GSTM1, The sequence of designed forward primer was: GAACCTCGAAAAGCTAAAGC and the reverse primer was: GTTGGGCTCAATATACGGTGG and these primers could amplify a nucleotide sequence of 219 base pairs region of GSTM1 gene. Also for the GSTT1, The sequence of designed forward primer was: TTCCCTACTGGTCTTCATCTCTC and reverse primer was: TCACCCATCATGGCCACGCA which amplify a nucleotide sequence of 480 base pairs region of GSTT1 gene.

For confirming PCR and detecting null genes of GSTT1 and GSTM1 we used a pair of Beta-Globin primers as a control gene (Housekeeping gene) which the forward primer sequence was: CAACCATCCACGTCACC, and the reverse primer sequence was: GAAGAGCCAGACAGGTA. They amplified a sequence of 268 base pairs region of the beta-globin gene.

PCR reaction was done in 0.2 ml micro tubes containing 10X PCR buffer with 0.75 µl of 50 Mm magnesium chloride and 0.5 µl of dNTP (0.1 mM). Also 0.3 µl from each of GSTT1, GSTM1 and Beta-Globin primers and 15 µl DNA polymerase and 2 µl DNA samples were added. Then the mixture was attenuated to final volume of 20 µl. PCR reaction was done for 35 cycles in thermocycler.

Agarose Gel Electrophoresis

PCR products were extracted during 120 minutes and using agarose gel 2.5% by 110 volts and then stained with ethidium bromide (13 g/ml). DNA fragments reached by GSTT1, GSTM1 and Beta-globin genes had 219, 480 and 268 base pairs (Figure 1).

Statistical Analysis

The data of gene polymorphism and sperm specifications were entered and organized in the Microsoft Excel program, then transferred to the SPSS. All statistical analysis was done by IBM SPSS software (Version 19.0) on the data. T-test was used to compare frequency of null and positive GSTT1 and GSTM1 genotypes between study groups. P-value <0.05 was considered as significant level.
Results
Totally 94 participants (46 patients with varicocele and 48 healthy men as a control) were included in the study. Mean age of participants was 30.77±6.46 in case and 32.74±4.94 in control groups. Results of sperm analysis, including sperm count, morphology and motility (fast and slow) are indicated in tables 2 and 3 for GSTT1 and GSTM1 genes, respectively. The difference between sperm count, morphology and fast motility was higher in control group significantly (P<0.05) while in slow motility difference was not significant (P>0.05).

Table 1. Effect of GSTT1 deletion on sperm parameters in groups of study (t-test).

<table>
<thead>
<tr>
<th>Group</th>
<th>Sperm parameter</th>
<th>Case</th>
<th>Control</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GSTT1 (−)</td>
<td>GSTT1 (+)</td>
<td>GSTT1 (−)</td>
<td>GSTT1 (+)</td>
</tr>
<tr>
<td>Count (million/ml)</td>
<td>71.77</td>
<td>78.29</td>
<td>105.20</td>
<td>98.95</td>
</tr>
<tr>
<td>Morphology (percent)</td>
<td>23.13%</td>
<td>25.87%</td>
<td>46.54%</td>
<td>51.83%</td>
</tr>
<tr>
<td>Fast motility (grade A)</td>
<td>13.68</td>
<td>12.20</td>
<td>22.91</td>
<td>27.75</td>
</tr>
<tr>
<td>Slow motility (grade B)</td>
<td>31.09</td>
<td>34.66</td>
<td>31.16</td>
<td>36.87</td>
</tr>
</tbody>
</table>

Results show that frequency of GSTM1 null genotype in the case and control groups were 60.9% and 41.7% (P=0.06) While the GSTT1 null genotype was 47.8% and 50% in case and control groups respectively (P=0.83).

Out of 46 patients with varicocele 22 patients had null GSTT1 genotype (47.8%) and 24 patients (52.2%) had not any deletion in this gene. From 48 healthy controls 24 (50%) had null and positive GSTT1 genotypes that there was no significant difference between groups (P=0.83). About GSTM1 from 46 patients in case group 28 patients (60.9%) had null and 18 (39.1%) had positive (no deletion) genotypes (0.06). In case group 16 men (34.8%) and 10 men (20.8%) of the control group had deletion in both GSTM1 and GSTT1 genes (P=0.18).

Table 2. Effect of GSTM1 deletion on sperm parameters in groups of study (t-test).

<table>
<thead>
<tr>
<th>Group</th>
<th>Sperm parameter</th>
<th>Case</th>
<th>Control</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GSTM1 (−)</td>
<td>GSTM (+)</td>
<td>GSTM1 (−)</td>
<td>GSTM1 (+)</td>
</tr>
<tr>
<td>Count (million/ml)</td>
<td>78</td>
<td>70.77</td>
<td>83</td>
<td>115.71</td>
</tr>
<tr>
<td>Morphology (percent)</td>
<td>24%</td>
<td>25.44%</td>
<td>43.8%</td>
<td>53.03%</td>
</tr>
<tr>
<td>Fast motility (grade A)</td>
<td>12</td>
<td>14.33</td>
<td>19.75</td>
<td>29.32</td>
</tr>
<tr>
<td>Slow motility (grade B)</td>
<td>32.57</td>
<td>33.55</td>
<td>30.50</td>
<td>36.53</td>
</tr>
</tbody>
</table>

Analysis of sperm parameters (count, morphology and motility) between participants with GSTT1 null and positive genotypes did not show significant differences (P>0.05). Also, there was no significant difference between GSTM1 null and positive genotypes according to motility and morphology (P>0.05) but in men with varicocele and null GSTM1 genotype sperm count was lower significantly (P=0.03) (Table 1 and Table 2).

Discussion
Varicocele is one of the most prevalent urologic diseases in men and also is an important cause of infertility. The exact cause of varicocele remained unknown yet. Several studies suggested oxidative stress induced by free oxygen radicals as an influencing factor in varicocele pathogenesis. It is proved that semen contains a certain amount of glutathione-S-transferase (GST) that can decrease toxic effects of free oxygen radicals in human sperm.
GST attaches glutathione to toxin which helps better solubility and facilitates cells to be cleaned of toxins (Chen et al., 2001, Wu et al., 2009, Acar et al., 2012). GST expression could be induced by exposure to an external substrate at in vivo environment. This shows that GST is a part of human immune system against chemical stresses (Chen et al., 2001, Wu et al., 2009, Acar et al., 2012).

GSTT1 and GSTM1 have expressed in different shapes. This gene is deleted in some people, which can be detected by somatic cells PCR-DNA diagnostic tests. It is shown that in people with GSTM1 and GSTT1 deletions related enzymes have no functions. GSTM1 and GSTT1 deletion has been reported between 10%-60% of people in different regions and races (Arruda et al., 1998).

Prevalence of the deleted genotype of GSTM1 has been reported about 50% in Caucasians and 20%-48.9% in other different races (Arruda et al., 1998, Rossini et al., 2002, Naveen et al., 2004). Also prevalence of genotype deletion of GSTT1 has been reported in 10%-20% of Caucasians (Norppa, 2001) and 16.8%-25% of other races (Arruda et al., 1998, Rossini et al., 2002, Naveen et al., 2004). Anyway, these loci are not attached and deletion of one is not necessarily attached with another one deletion (Acar et al., 2012). Considering with higher prevalence of GSTM1 genotype deletion its polymorphism in infertility has been considered more. In a case-control study in Turkey, there were no significant differences between groups according to the prevalence of deletion of GSTT1 and GSTM1 genes (Chen et al., 2002).

In another study on GSTM1 polymorphism in infertile men with varicoceles in Taiwan, no significant difference was found between healthy controls and patients with varicocele (Nelson et al., 1995). Also a study in Iran had similar results according to GSTM1 and GSTP1 genes polymorphism (Mirfeizollahi et al., 2009). 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).

Another study in China showed that oxidative damages could be the cause infertility in varicocele patients and null genotype of GSTT1 will increase risk of oxidative damages in such patients (Arruda et al., 1998).

As we see the results of our study is concomitant with other previous studies and shows that GSTT1 and GSTM1 polymorphism has no considerable effect on sperm parameters. Differences in some results possibly are due to different races or some other factors that caused infertility in these study men and had been missed. But it seems that considering with frequent enzymes related to GST it is possible that other loci of this gene family have compensated absence of GSTM1 and GSTT1 related enzymes effects and future studies should focus on these genes effects and polymorphisms and also their effects on sperm parameters in patients with varicocele.

In conclusion of our research, deficiency of enzyme activity in GSTM1 null genotype were not affected on morphology, slow and quick progressive of sperm, but caused the significant decrease in count of sperm between GSTM1 null and positive genotype. In the case of GSTT1, the effect of GSTT1 null genotype, no affected on sperm parameters that compensate activity of this super family gene may justify the cause. Future studies should focus on possible compensatory effects of other GST enzymes family and their polymorphism.
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