

The association of seminal plasma antioxidant levels and sperm chromatin status with genetic variants of *GSTM1* and *GSTP1* (Ile105Val and Ala114Val) in infertile men with oligoasthenoteratozoospermia

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Abstract. In this study we aimed to examine the effects of genetic variants of *GSTM1* and *GSTP1* (Ile105Val and Ala114Val) on GST activity, seminal oxidative stress and sperm chromatin status in infertile men with oligoasthenoteratozoospermia (OAT). The study population ($n = 121$) consisted of 95 infertile men with OAT and 26 controls with normozoospermia. Multiplex polymerase chain reaction (PCR) and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) methods were utilized to detect the aforesaid genetic variants. We measured GST activity and total antioxidant capacity (TAC) of seminal plasma by spectrophotometry. Sperm chromatin integrity and maturity were assessed using toluidine blue and chromomycin A₃ (CMA₃-positive sperm) staining, respectively. The analysis showed that subgroups of *GSTM1* null and *GSTP1* C/T+T/T genotypes in comparison with *GSTM1* present and *GSTP1* wild type (C/C) genotypes did not have statistically significant differences in both OAT or normozoospermic men considering sperm concentration and motility, percentage of CMA₃-positive sperm, seminal plasma TAC, sperm chromatin integrity and GST activity. Thus, the findings of our study suggest that there are no significant associations between *GSTM1* and *GSTP1* polymorphisms and sperm parameters at conventional or at molecular levels including OS status, sperm chromatin integrity or maturity in Iranian infertile men with OAT and normozoospermia. However, these polymorphisms could be related to the fertility status of the studied population but not evaluated in this study.

Keywords: *GSTM1*, *GSTP1*, normozoospermia, polymorphism, oligoasthenoteratozoospermia, sperm

1. Introduction

Redox balance is disrupted due to imbalances in reactive oxygen species (ROS) production and their scavenging, leading ultimately to oxidative stress (OS). OS

induces peroxidative damages to macromolecules in organs, tissues and cells, especially to polyunsaturated fatty acids in the cell membrane. In comparison with other cells, sperm has plenty of polyunsaturated fatty acids that make it prone to ROS-induced damages. Therefore, the protective mechanisms including antioxidant defense (enzymatic and non-enzymatic) against ROS are needed for both sperm and seminal plasma [1]. One of the enzymatic antioxidants in human sperm and seminal plasma is glutathione S-transferase (GST) that

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detoxifies products of lipid peroxidation such as aldehydes, alkanes, hydroperoxides and epoxides generated in the cell membrane. GST has at least six isoenzymes, among which implication of *GSTM1* and *GSTP1*, in fertility-related events has been noted [2]. *GSTM1* and *GSTP1* isoenzymes, which encode mu and pi proteins, respectively, are found on the surface of human spermatozoa [3]. These two genes have important functional polymorphisms in different racial and ethnic populations. A deletion in *GSTM1* gene locus (null genotype) leads to the production of a protein with no enzymatic activity [4]. In men with unexplained infertility, the null genotype of *GSTM1* increased peroxidative damage to sperm membrane and chromatin [5]. Similar results were obtained in infertile men with varicocele [6].

Two *GSTP1* polymorphisms in exon 5 (Ile105Val) and exon 6 (Ala114Val) have been reported [7]. These variants in the active site of *GSTP1* are accompanied with reduction in enzyme activity [8].

Despite the importance of various GST genotypes in different diseases and their differential distribution in different populations, studies focusing on the role of GST on normal function of spermatozoa and its impairment in male sub/infertility are rather limited. Therefore, we conducted the current study to investigate the relation between seminal plasma antioxidant concentrations, sperm chromatin status, GST enzyme activity and genetic variants of *GSTM1* and P1 in Iranian infertile men with severely compromised semen parameters or oligoasthenoteratozoospermia (OAT) in comparison with normozoospermic control groups.

2. Materials and methods

2.1. Patients

Semen and blood samples were obtained from men who were referred to Avicenna Infertility Clinic (AIC) in Tehran, Iran. The study sample size consisted of 121 men, which included 95 men with oligoasthenoteratozoospermia (OAT) and 26 with normozoospermia. According to WHO criteria, men were identified to have OAT if the total sperm motility was < 50%, sperm count < 20×10^6 /ml and sperm with normal shapes was < 30% [9].

Men with leukocytospermia ($WBC \geq 1 \times 10^6$ /ml), genital tract infection, congenital anomalies, high-grade varicocele, alcohol consumption, hypogonadism, history of cryptorchidism, chronic systemic disease, liver disease and positive sperm mixed-agglutination

reaction test (MAR test) were excluded from the study. This study was approved by the Ethics Committee of Avicenna Research Institute, in Tehran, Iran. Written informed consent was obtained from each participant before the study.

2.2. Sample collection and preparation

Semen samples were obtained by masturbation after at least 48 to 72 h of sexual abstinence. Semen analysis was performed in accordance with WHO guidelines [9]. Each sample was aliquoted into 2 parts; part 1 was freshly used for the assessment of sperm chromatin integrity and maturity and GST activity assays, while part 2 was centrifuged at 1800 rpm for 10 minutes to get clear seminal plasma for total antioxidant capacity (TAC) assay. The resultant supernatant was re-centrifuged at 4000 rpm for 10 minutes at room temperature to remove remnants of spermatozoa and cell debris. The supernatants were stored at -70°C for later TAC assay. Peripheral blood samples (~ 5 ml) were collected from all individuals in EDTA-containing tubes and preserved at -20°C for later DNA extraction.

2.3. Assessment of sperm chromatin integrity and maturity

Sperm chromatin integrity and maturity were evaluated by toluidine blue (TB) and chromomycin A₃ (CMA₃-positive sperm) staining tests as described by Lakpour et al. [10].

2.4. Assessment of GST activity and TAC

The glutathione-S-transferase activity was determined as previously described [11,12]. The reaction solution was constructed by mixing 980 μl of 0.1 M PBS, 10 μl of 100 mM GSH and 10 μl of 100 mM 1-chloro-2,4-dinitrobenzene (CDNB). In the next step, 900 μl of the aforesaid solution was added to 100 μl of sperm suspension (1×10^6 sperm). Reading the absorbance at 340 nm in a spectrophotometer thermostated at 37°C , we calculated the difference in optical absorbance (ΔA) per min and reported GST activity as nmol/min/ 1×10^6 sperm.

Seminal plasma TAC was assessed by a previously described method of using a decolorization assay [13].

2.5. *GSTM1* and *GSTP1* polymorphisms

Genomic DNA was extracted from peripheral blood lymphocytes by salting out procedure, according to Miller et al. [14]. The presence or absence of *GSTM1* gene was determined using multiplex polymerase chain reaction (PCR); however analysis of *GSTP1* gene polymorphism was performed by polymerase chain reaction restriction fragment length polymorphisms (PCR-RFLP). *GSTM1* promoter fragments from each individual were amplified using forward primer: 5'-GAACTCCCTGAAAAGCTAA-3' and reverse primer: 5'-GTTGGGCTCAAATA TACGGTGG-3'. β -globin primers (forward, 5'-CAACTTCATCCAC GTTACC-3' and reverse, 5'-GAAGAGCCAAGGAC A GGTAC-3') were used as internal control for DNA amplification.

The multiplex PCRs were carried out in a total volume of 20 μ l containing 10 mmol/l Tris-HCl (pH 8.3), 0.2 mmol/l MgCl₂, 50 mmol/l KCl, 0.2 mmol/l each dNTP, 0.175 pmol/ μ l β -globin primers, 0.975 pmol/ μ l *GSTM1* primers, 0.05 U of Taq DNA polymerase (Roche) and 100 ng of template DNA. *GSTM1* PCR was performed for 35 cycles in a DNA thermal cycler using a thermal profile of denaturation at 94°C for 20 s, annealing at 60°C for 20 s, and primer extension at 72°C for 30 s. *GSTP1* primers included forward, 5'-CTATGGGAAGGACCAGCAGG-3' and reverse, 5'-TGGTCTCCACAATG-3'. *GSTP1* PCRs were carried out in a total volume of 25 μ l containing 10 mmol/l Tris-HCl (pH 8.3), 1.5 mmol/l MgCl₂, 50 mmol/l KCl, 0.4 mmol/l each dNTP, 0.28 pmol/ μ l *GSTP1* primers, 0.04 U of Taq DNA polymerase (Roche) and 100 ng of template DNA. This PCR was performed for 30 cycles in a DNA thermal cycler using a thermal profile of denaturation at 94°C for 30 s, annealing at 58.9°C for 30 s, and primer extension at 72°C for 40 s. The PCR products for *GSTM1* and β -globin were separated on a 2.5% agarose gel at 80 V for 2 h and stained with 1 μ g/ml ethidium bromide. DNA fragment sizes of the *GSTM1* and β -globin genes amplified by PCR were 215 bp and 268 bp, respectively.

Individuals with null (-) genotype of *GSTM1* did not have 215 bp band while they did have the 268 bp band. The presence of PCR product for *GSTP1* with a size fragment of 1069 bp was confirmed on 1.5% agarose gel following staining by ethidium bromide (1 μ g/ml). Subsequently, *GSTP1* PCR product (1069 bp) was digested by AccI and FauI for evaluating *GSTP1* polymorphisms, 313 A \rightarrow 313 G in exon 5 and 341C>T in exon 6, respectively. The digestions were carried out in

Table 1

Demographic characteristics of infertile men included in this study

Group	Normozoospermia	OAT	P-value
Age (year)	37.19 \pm 6.57	36.26 \pm 8.12	NS
Infertility duration (year)	4.48 \pm 5.25	5.32 \pm 7.39	NS
Smoking status	2 (9.52%)	15 (17.04%)	NS

OAT, oligoasthenoteratozoospermia; NS, not significant.

Table 2

The frequency of *GSTM1* and *P1* genotypes in OAT and normozoospermic men

Group	Normozoospermia N (%)	OAT N (%)	P-value	OR (95% CI)
<i>GSTM1</i> genotype				
Null	14 (53.8)	49 (52.1)	-	-
Positive	12 (46.2)	45 (47.9)	0.877	0.933
<i>GSTP1</i> genotype				
C/T+T/T	3 (11.5)	18(18.9)	-	-
C/C	23 (88.5)	77 (81.1)	0.377	0.558

OAT, oligoasthenoteratozoospermia; OR, odds ratio; 95% CI, 95% confidence interval.

a total volume of 10 μ l, once containing 1 μ l of NEB-buffer (Biolabs, England), 0.3 μ l of AccI (10000 U/ml Biolabs), and 5 μ l of PCR product for *GSTP1* 313 A>G and once containing 1 μ l of NEBuffer (Biolabs), 0.5 μ l of FauI (2000 U/ml Biolabs), 3.5 μ l of PCR product for *GSTP1*341C>T.

The PCR product and its digested products were separated by electrophoresis on 10% polyacrylamide gel and were subsequently stained using silver nitrate [15]. The men with wild type (313 A/A) *GSTP1* had a single 1069-bp band, those with heterozygous (313 A/G) had three 1069 bp, 982 bp and 87 bp fragments and homozygote men (313 G/G) had two 982 bp and 87 bp fragments. Participants with wild type (341 C/C) *GSTP1* had three 602 bp, 365 bp and 102 bp fragments, heterozygotes (341 C/T) had three 1069 bp, 602 bp, and 467 bp fragments and those with homozygote genotype (341 T/T) had two 602 bp and 467 bp fragments.

2.6. Statistical analysis

First, distribution of the variables was determined using one-sample Kolmogorov-Smirnov test. The comparison of frequencies for *GSTM1* and *GSTP1* genotypes between groups were done by chi-square test. To compare parameters with normal distribution, we used independent sample *t* test and for parameters with non-normal distribution, we used Mann-Whitney *U* test. We considered *P* < 0.05 as statistically significant.

Table 3

GST polymorphisms in relation to sperm concentration, sperm motility, sperm normal morphology, percentage of CMA₃-positive sperm, seminal plasma TAC, sperm chromatin damage and GST enzyme activity

Group	Concentration	Motility	NM	CMA ₃ -ps	TAC	SCD	GSTA
Normozoospermic men							
<i>GSTM1</i>							
Null	133.53 ± 69.15	54.23 ± 23.79	28.77 ± 9.65	28.16 ± 14.52	7.76 ± 1.13	35.77 ± 20.01	7.39 ± 1.68
Positive	138.47 ± 125.04	51.25 ± 9.32	26 ± 9.33	33.45 ± 19.09	7.76 ± 0.91	31.75 ± 19.76	6.7 ± 1.7
<i>GSTP1</i>							
C/T+T/T	143.17 ± 99.91	52.5 ± 19.25	28.82 ± 8.79	31.9 ± 17.61	7.72 ± 1.08	32.85 ± 20.11	7.08 ± 1.69
C/C	82.66 ± 71.49	55	17.33 ± 8.73 [#]	22.66 ± 2.59	7.93 ± 0.4	38.66 ± 18.23	6.95 ± 2.18
OAT men							
<i>GSTM1</i>							
Null	31.93 ± 15.97	42.51 (35–50)	9.26 ± 7.21	40.68 ± 20	7.7 ± 1.26	35.08 ± 17.38	7.1 ± 2.53
Positive	31.66 ± 18.9	40 (16.75–50)	8.75 ± 7.2	38.02 ± 16.27	7.74 ± 1.24	33.59 ± 17.54	7.47 ± 2.9
<i>GSTP1</i>							
C/T+T/T	30 ± 14.46	40 (30–50)	8.76 ± 7.06	39.43 ± 19.04	7.76 ± 1.29	33.87 ± 17.87	7.16 ± 2.55
C/C	38.28 ± 25.33	42.5 (26.25–50)	9.56 ± 7.89	39.87 ± 16.1	7.64 ± 1.59	36.46 ± 15.61	7.88 ± 3.39

GST: glutathione S-transferase; TAC: total antioxidant capacity; NM: sperm normal morphology; CMA₃-ps: percentage of CMA₃-positive sperm; SCD: sperm chromatin damage; GSTA: glutathione S-transferase enzyme activity. [#]: Compared to the *GSTP1* C/T+T/T group in normozoospermic men, $p = 0.045$. Data were presented in median (25th–75th percentile) and mean ± SD.

3. Results

In view of age, there were no significant differences between men with OAT and normozoospermia. Other demographic characteristics and the frequencies for *GSTM1* and *P1* genotypes in men with OAT and normozoospermia have been presented in Tables 1 and 2, respectively.

The frequency of *GSTM1* null genotype was 52.1% in men with OAT and 53.8% in men with normozoospermia (OR = 0.933; 95% CI, 0.391–2.23; $P = 0.877$) and the frequency of *GSTP1* C/T+T/T genotypes were 18.9% in men with OAT and 11.5% in men with normozoospermia (OR = 0.558; 95% CI, 0.151–2.064; $P = 0.377$). Interestingly, all individuals in the two groups were A/A in +313 site (codon 105 of *GSTP1*). In the current study, there were no significant differences between men with OAT and normozoospermia with respect to the frequencies of *GSTM1* and *GSTP1* genotypes.

As expected, the total sperm motility ($P < 0.01$), sperm concentration and normal sperm morphology ($P < 0.001$) were significantly lower in men with OAT versus men with normozoospermia ($P < 0.001$). In addition, the percentage of CMA₃-positive sperm was lower in men with normozoospermic men than men with OAT. However, the two groups of men did not have significant differences with respect to sperm chromatin integrity, GST enzyme activity, and seminal plasma total antioxidant capacity (TAC). The characteristics of *GSTM1* and *GSTP1* polymorphisms regarding sperm concentration, sperm normal morphology, total sperm

motility, CMA₃-positive sperm, seminal plasma TAC, sperm chromatin integrity and GST enzyme activity have been shown in Table 3.

In contrast to men with OAT, the men with normozoospermia had sperm normal morphology significantly lower in the subgroup for wild type (C/C) *GSTP1* genotype than in the subgroup with the C/T+T/T *GSTP1* genotype ($P < 0.05$). However, sperm normal morphology was not significantly different in both OAT and normozoospermic men considering the *GSTM1* present or null genotypes.

In addition, sperm concentration and motility, percentage of CMA₃-positive sperm, seminal plasma TAC, sperm chromatin integrity and GST enzyme activities were not different in the subgroups of *GSTM1* null and *GSTP1* C/T+T/T genotypes versus the subgroups of *GSTM1* present and *GSTP1* wild type (C/C) genotypes in both groups of men with OAT and normozoospermia.

4. Discussion

Seminal oxidative stress created via over-production of ROS by means of white blood cells (WBCs), dead and immature spermatozoa is one of the important causes of male sub/infertility. Antioxidant defense systems, especially enzymatic ones, play a critical role in preventing or creation of OS. GSTs, as a member of enzymatic defense, attenuate OS through binding reduced GSH to toxic oxidants. Moreover, interactions between GST genes and deleterious environmental exposures have been addressed by exhibiting the presence of GST

on the head of spermatozoa and its role in detoxifying certain carcinogens [16].

GST gene polymorphisms and male sub/infertility is very dependent on ethnicity. Hence, the present study focused on genotypes of two isoenzymes of *GSTM1* and *GSTP1*. In *GSTP1*, substitution of Val instead of Ile and Ala in exon 5 (Ile105Val) and exon 6 (Ala114Val) significantly reduced enzyme activity. Therefore, simultaneous evaluation of these two polymorphisms in male infertility is a novel idea that has not been addressed yet.

According to our findings, frequency of *GSTM1* and *GSTP1* genotypes (both sites) were not significantly different between normozoospermic men and men with OAT. This finding had been previously confirmed in fertile men with good semen quality [17]. Olshan et al. showed that gene polymorphisms of *GSTM1*, *GSTT1*, and *GSTZ1* are not correlated with sperm quality. As a result, there seems to be no statistically significant relation between GSTs genotypes and changes in classical semen parameters in fertile and infertile men. Previous studies performed in different countries, including China (men with varicocele vs. fertile men; *GSTM1* and P1) [6], Turkey (men with varicocele vs. fertile men; *GSTM1*) [18], and Russia (idiopathic infertile men vs. fertile men; *GSTM1*) [19] have shown no significant differences between fertile men and men with varicocele or infertility. However, Tirumala, et al. indicated that the frequency of *GSTM1* null genotype is significantly lower in fertile than infertile men [20]. Additionally, Finotti et al. also showed that semen quality of men with idiopathic infertility decreases in *GSTM1/GSTT1* null genotypes [21]. Likewise, two more studies in the latter relation have been performed in Iran. The first, performed by Safarinejad et al., had surprising and unexpected results [22], but the second one, performed by Salehi et al., showed a correlation between idiopathic male infertility and *GSTT1* and *GSTM1* null deletions in men from Northern Iran [23]. Contrary to the study by Safarinejad et al. we found no individual with hetero- or homozygote genotypes in codon 105 of *GSTP1* (*GSTP1* 313 A/G and 313 G/G polymorphisms). For this reason, we checked the accuracy of restriction enzyme (AccI) activity by performing RFLP on the other nucleotide sequence that had a cutting site for AccI.

Contrary to our expectation, we found a positive correlation between sperm normal morphology and *GSTP1* C/T+T/T genotypes only in normozoospermic men. Although, sperm with normal morphology was higher in OAT men with *GSTP1* C/T+T/T genotypes

than in OAT men with *GSTP1* C/C genotype, but this difference was not statistically significant. In connection to this finding, two inferences can be suggested, the first *GSTP1* C/T+T/T genotypes are associated with sperm normal morphology (a protective role in normal spermatogenesis) and the second, increasing sample size in normozoospermic men could change this result. Therefore, any ultimate conclusions in this regard would be premature and further studies would be necessary.

Similar to Aydemir et al., we did not find any difference between *GSTM1* and *GSTP1* genotypes in total antioxidant capacity and GST enzyme activity [5]. Therefore, these polymorphisms seem not to affect GST activity and, consequently, antioxidant concentration in the seminal plasma of men with OAT or normozoospermic men.

Reduction of enzyme activity in one or two genotypes is compensated by other GST isoenzymes, including *GSTT1*, *GSTA1*, and *GSTM2*. Aydemir et al. found that *GSTM1* null genotype might negatively affect spermatozoa by increasing the oxidative stress in men with idiopathic infertility [5]. However, our findings did not confirm this concept in men with OAT or normozoospermia.

Since the studied polymorphisms did not affect classical sperm parameters, we decided to evaluate their effect on human spermatozoa at molecular level, including sperm chromatin integrity and maturity. In this regard, the present study showed sperm chromatin damage and maturity were not different in both men with OAT or normozoospermia; regarding *GSTM1* null and *GSTP1* C/T+T/T genotypes compared to *GSTM*-present and *GSTP1* C/C genotypes. Aydemir et al. mentioned that sperm is further susceptible to oxidative damage in idiopathic infertility with *GSTM1* null genotype [5]. In addition, Tang et al. showed that in men with varicocele, the *GSTM1* and *GSTT1* null genotypes lead to sperm oxidative damage but *GSTP1* alleles (codon 105) do not affect OS status of semen and, subsequently, oxidative damage of spermatozoa [6].

Finally, it should be mentioned that our study had some limitations. First, we did not cover fertile men in our study. Moreover, the normozoospermic group had a small sample size. Additionally, TAC-ROS score could show OS status better than TAC.

5. Conclusion

In conclusion, the findings of this study suggests that there are no significant associations between *GSTM1*

and *GSTP1* (codon of 114) polymorphisms and sperm parameters at classical or at molecular levels, including OS status, sperm chromatin integrity or sperm chromatin maturity in male Iranians with OAT and normozoospermia. However, these polymorphisms may be related to the fertility status of the studied men not evaluated in this study.

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