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Relationships Between Levels of Reactive Oxygen Species in Spermatozoa and GSTM1 Polymorphism in Infertile Men

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Abstract

The purpose of this study was to examine relationships between glutathione S-transferase Mu-1 (GSTM1) gene polymorphism and the susceptibility of spermatozoa from patients with idiopathic infertility to oxidative stress. Fifty-two men with idiopathic infertility and 60 healthy fertile men were recruited to this study. GSTM1 gene polymorphism was determined by polymerase chain reaction (PCR) and both the infertile and control individuals were divided into GSTM1 null and GSTM1 positive groups according to their GSTM1 gene structure. ROS formation in spermatozoa was followed by chemiluminescence of luminol-fluoresceine-enhanced system. We compared reactive oxygen species (ROS) generation in spermatozoa from infertile patients and controls with respect to GSTM1 genotype. Significantly higher levels of ROS were found in idiopathic infertile men with the GSTM1 null genotype compared with those with the GSTM1 positive genotype. There was no significant difference in genotype distribution for the GSTM1 variant between the idiopathic infertile subjects and fertile subjects. Our results suggest that the susceptibility of spermatozoa to oxidative stress is significantly greater in idiopathic infertile men with the GSTM1 null genotype compared with those possessing the gene. Therefore, in patients with idiopathic infertility, GSTM1 polymorphism might be an important source of variation in susceptibility of spermatozoa to oxidative damage.

Key words: GSTM1 polymorphism, Reactive Oxygen Species, Spermatozoa

INTRODUCTION

Oxidative stress is believed to underlie the etiology of numerous human conditions. Organisms are subject to oxidative stress from endogenous and exogenous sources including exposure to solvents, other chemicals and environmental pollutants. All these potential hazards contain components that can induce severe macromolecular, cellular and tissue damage through a) direct cytotoxic effects, b) promotion of primary genotoxic events, or c) generation of reactive oxygen intermediates [1]. Reactive oxygen species (ROS), such as the superoxide anion and hydroxyl radical, can be produced by human spermatozoa [2,3]. As a result of a high polyunsaturated fatty acid content, human spermatozoa plasma membranes are highly sensitive to ROS-induced damage and hydrogen peroxide appears to be the most toxic ROS for human spermatozoa. Oxidative stress is induced by ROS. Normal levels of ROS are required for sperm physiology, but excessive levels of ROS can negatively affect sperm quality. The oxidative stress-induced sperm damage has been suggested to be a significant contributing factor in 30–80% of all cases of male infertility. The generation of ROS can be exacerbated by environmental, infectious, and lifestyle etiologies (Figure 1) [3]. For example, exposure to cigarette smoke generates high levels of oxidative stress, directly increasing seminal leukocyte concentrations and seminal ROS generation, and decreasing seminal levels of the antioxidant enzyme superoxide dismutase (SOD) [3]. There is growing evidence that peroxidative damage to the human spermatozoa membrane is an important pathophysiological mechanism in human male infertility [3, 4]. Human spermatozoa and seminal plasma possess various antioxidant systems to scavenge ROS and prevent ROS-related cellular damage [1-4]. Failure of antioxidant defences to detoxify excess ROS production can lead to significant oxidative damage including enzyme inactivation, protein degradation, DNA damage and lipid peroxidation. These antioxidant defense systems, which are involved in a variety of detoxification reactions, exhibit baseline levels of activity to ensure the maintenance of the balance between production and removal of endogenous ROS and other pro-oxidants [1].

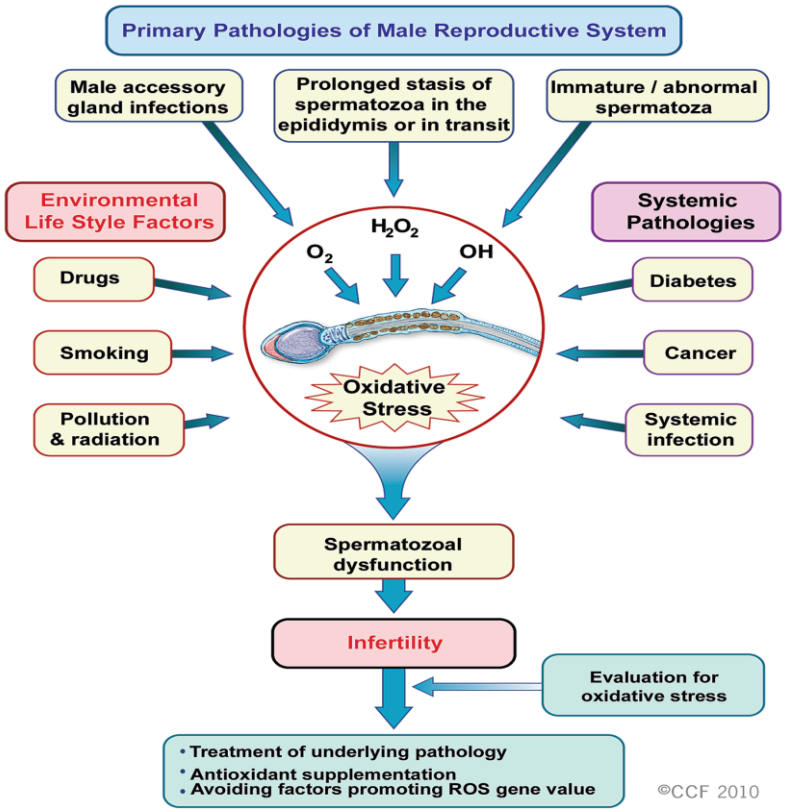

Figure 1. Relationship of the primary pathologies of the male reproductive system, oxidative stress and infertility [3].

One of the defense systems against the damaging effects of oxidative stress in human semen are the glutathione S-transferases (GST; EC 2.5.1.18), which catalyze the conjugation of glutathione (GSH) with various electrophilic substances, and play a role in preventing oxidative damage by conjugating breakdown products of lipid peroxides to GSH [5]. It is known that GST activity is widely distributed in hepatic and extrahepatic tissues including the ovaries, testes and serum, and it has been shown that GST might have a relevant protective role during spermatogenesis [6].

GST have been grouped into at least six classes called Alpha, Mu, Pi, Theta, Sigma and Zeta. Genes encoding the glutathione S-transferase Mu-1 and Theta-1 (GSTM1 and GSTT1, respectively) isoforms are polymorphic. Homozygotes for the mutated inactive alleles of each gene are devoid of any specific enzymatic activity (null genotypes). Up to 50% of the Caucasian population are null genotypes for the GSTM1 gene. The GSTM1 gene deletion might, therefore, modify the risk of individuals to expose to toxins. Several epidemiological studies have reported that the GSTM1 null genotype is correlated with an increased susceptibility to diseases associated with oxidative stress and proposed that GSTM1 might be a critical isozyme in the detoxification of oxidative stress products [7-12]. Chen et al. [8] have shown that sperm of varicocele patients with the GSTM1 null genotype are more vulnerable to oxidative damage. It has been also reported that seminal plasma and spermatozoa from men with idiopathic infertility have higher ROS levels than those from the fertile men [13]. If enzymatic deficiency in the GSTM1 isoform is correlated with increased risk of certain diseases associated with oxidative damage, then it is possible that there is an association between GSTM1 genotypes and idiopathic infertility. Several studies reported that the potential role of different detoxification mechanisms such as superoxide dismutase, catalase, GSH peroxidase and GSH have been investigated in idiopathic infertility [13, 14].

Therefore, the general aim of the present study was to determine whether the GSTM1 null genotype is associated with altered susceptibility to oxidative stress, and the ROS production of sperm in patients with idiopathic infertility.

MATERIALS AND METHODS

Collection and preparation of samples

The present study was approved by the institutional review board of the Infertility Central Urology Department of Cerrahpaşa Medical Faculty, Istanbul, Turkey. Blood and semen specimens were obtained from 52 men aged 26-49 years with idiopathic infertility. Specimens were also obtained from 60 male volunteers aged 25-50 years with normal semen analysis according to World Health Organization (WHO) guidelines [15] to serve as the fertile control. The Institutional Ethical Committee approval was taken in accordance with the principles of Declaration of Helsinki. Informed consent was obtained from each study subject. Individuals with a significant medical history or signs suggestive of defective androgenisation or abnormal testicular examinations were excluded from this study. Further exclusion criteria for both groups included chromosomal disorders related to a fertility disorder, cryptorchidism, vasectomy, anormal liver function and hormone tests, cigarette smoking, alcohol consumption and the use of folic acid, glutathione, vitamin C, vitamin E supplements or medication within three months before recruitment. Criteria for study inclusion were infertility for at least 12 months with at least one semen parameter abnormality, semen leukocyte count less than 1×10^6/mL

and negative semen antisperm antibody on a mixed agglutination reaction test. Semen specimens were collected by masturbation into a sterile wide-mouth metal-free plastic container after at least 3 days (3-5 days) of abstinence and liquified at 25ºC for 30 min.

Semen analysis

A semen analysis was carried out according to the WHO guidelines to obtain volume, pH, sperm concentration, motility and morphology. Sperm concentration was determined using a Makler Counting Chamber (Seti-Medical Instruments, Haifa, Israel). Motility was expressed as the percentage of motile spermatozoa and their mean velocity. Morphology was determined according to the WHO criteria after incubation of the sample with trypsin for 10 min at 25ºC, using the methylene blue eosin staining procedure, feathering and fixation by flame. At least 100 cells were examined at a final magnification of × 1 000 [15].

Spermatozoa preparation

After liquefaction, spermatozoa were fractionated on Percoll gradients (40-95%) according to WHO guidelines [15]. Semen was layered on top of the gradient and centrifuged at 400 × g for 20 min at 25ºC. Spermatozoa in the 95% Percoll layer were collected, and washed twice at 400 × g for 6 min at 25ºC with added Tris, sodium and EDTA (TNE) buffer (0.15 mol/L NaCl, 0.01 mol/L Tris-HCl, 1 mmol/L Na_2EDTA, pH 7.4) [13]. ROS levels within the spermatozoa were determined immediately after washing.

Measurement of ROS

ROS were measured in spermatozoa, immediately after collection and washing, using a luminol (5-amino-2, 3,-dihydro-1, 4-phthalazinedione)-enhanced chemiluminescence method [13, 16]. Luminol was prepared as 5 mmol/L stock in dimethyl sulfoxide (DMSO). 10 µL of the stock was added to 500 µL of the sperm suspension (1 × 106 spermatozoa/mL). Negative control was prepared by adding an equal amount (10 µL) of luminol to 500 µL of TNE buffer. The basal levels of ROS in spermatozoa were assessed by measuring the luminol-dependent chemiluminescence with the Luminoskan TL luminometer (Labsystems Inc., Helsinki, Finland) in the integrated mode for 10 min. After stimulation with 25 µM cumene hydroperoxide (CumOOH) and 30 µM benzo[a]pyrene (BaP) for 10 min, the levels of ROS production in spermatozoa were measuring the same system. Results were expressed as relative light units (RLU) per 1×10^6 spermatozoa/mL.

GSTM1 polymorphism

For the determination of the genetic status, DNA was prepared from peripheral lymphocytes of anticoagulated blood (EDTA) by proteinase K digestion and a salting out procedure with a saturated NaCl solution described by Miller et al. [17]. The polymerase chain reaction (PCR) method was used to detect the presence or absence of the GSTM1 gene as described previously [18]. The GSTM1 primers used were: forward, 5'-GAACTCCC TGAAAAGCTAAAGC-3'; reverse, 5'-GTTGGGCTCAA ATATACGGTGG-3'. The b-globulin primers used were: forward, 5'-CAACTTCATCCACGTTCACC-3'; reverse, 5'-GAAGAGCCAAGGACAGGTAC-3'. Polymerase chain reaction was carried out for 35 cycles in a DNA thermal cycler using a thermal profile of denaturation at 94ºC for 1 min, annealing at 55ºC for 1 min and primer extension at 72ºC for 1 min. The PCR products were then separated on a 2% agarose at 150 V for 1.5 h, and stained with 1 µg/mL ethidium bromide at 25ºC for 10 min. DNA from individuals with positive GSTM1 and b-globulin alleles yielded 215- and 268-bp products, respectively. The absence of amplifiable GSTM1 (in the presence of b-globulin PCR product) indicates a GSTM1 null genotype. The presence of amplifiable GSTM1 indicates positive genotype (homozygous or heterozygous for the GSTM1 gene). (Show the data).

Values reported are mean ± SD. All data were normally distributed and underwent equal variance testing. Statistical significance of differences was determined by SPSS version 17.0 for windows (SPSS, Chicago, IL, USA). Statistical analysis was performed by Mann-Whitney U-test and One Way ANOVA with Tukey's post test. $P < 0.05$ was considered statistically significant.

RESULTS

There was no statistically significant difference in frequency of the GSTM1 null genotype between the idiopathic infertile group (51.9 %) and the control group (53.3 %). Furthermore, no statistical differences between idiopathic and fertile males were found for sperm morphology in semen analysis (Table 1). Sperm concentrations were significantly lower in the patients with idiopathic infertile men compared with individuals in the control group ($p<0.001$). Additionally, sperm concentrations were lower in GSTM1 positive and null patients with idiopathic infertile men compared with the GSTM1 positive and null control subjects (for each, $p<0.001$). No such differences in sperm concentrations were noticed between GSTM1 positive and null control subjects. Sperm motility were significantly lower in GSTM1 null patients with idiopathic infertile men compared with GSTM1 positive genotype controls ($p<0.05$).

Levels of ROS were measured in washed sperm suspansions, using chemiluminescence assay. Basal levels of ROS were measured in washed sperm suspensions, using chemiluminescence assay. Basal and induced by CumOOH and BaP ROS levels in the spermatozoa samples were also significantly higher in specimens from infertile patients than that from controls (for each, $p < 0.001$) (Table 2). Significantly higher levels of ROS were also found in idiopathic infertile men with the GSTM1 null genotype compared with those with the GSTM1 positive genotype ($p < 0.001$). Additionally, basal levels of ROS were significantly higher in individuals from GSTM1 null genotype control than that from GSTM1 positive genotype controls ($p< 0.001$). Following supplementation with BaP or CumOOH, time-dependent increases were observed in the production of ROS after incubation for 10 min. Significantly higher levels of ROS production were also found in idiopathic infertile men with the GSTM1 null genotype compared with those with the GSTM1 positive genotype and control subjects GSTM1 null and positive genotypes (for each, $p<0.001$).

Table 1. The population semen variebles in idiapathic infertile men and controls in terms of *GSTM1* genotype.

Groups	Sperm count ($x10^6$/ml)	Sperm motility (%)	Sperm morphology (%)
Total Patients Group (n=52)	27.44±5.47[e]	47.06±1.47	47.35±1.55
Patient *GSTM1* null (n=27)	25.89±3.38	46.67±1.41	47.19±1.82
Patient *GSTM1* positive (n=25)	29.12±6.75[b,c]	47.48±1.45	47.52±1.19
Total Control Group (n=60)	56.08±7.48	47.45±1.27	47.48±1.28
Control *GSTM1* null (n=32)	54.88±7.03[a]	47.22±1.34	47.25±1.41
Control *GSTM1* positive (n=28)	57.46±7.86[a]	47.71±1.15[d]	47.75±1.08

Values of semen variebles between GSTM1 positive and GSTM1 null genotypes in idiopathic infertile patients and control subjects by One Way ANOVA, Post Hoc Tukey. Data were presented as Mean ±S.D. [a]When compared to patient GSTM1 null group, p<0.001; [b] When compared to control GSTM1 positive group, p<0.001; [c] When compared to control GSTM1 null group, p<0.001; [d]When compared to patients GSTM1 null group, p<0.05; [e]When compared to control group, p<0.001 (Mann-Whitney U-test).

Table 2. Basal levels of ROS (RLU) and induced by CumOOH and benzo[a]pyrene ROS formation levels in spermatozoa of idiopathic infertile men and control subjects with respect to *GSTM1* genotype.

Groups	Basal level	25 µM CumOOH	30 µM Benzo[a]Pyrene
Total Patients Group (n=52)	25.23±14.54[d]	42.15±11.27 [d]	83.92±50.82 [d]
Patient *GSTM1* null (n=27)	38.67±4.35	50.41±7.89	131.70±10.43
Patient *GSTM1* positive (n=25)	10.72±2.56[a,b]	33.24±6.57 [a,b]	32.32±5.38 [a,b]
Total Control Group (n=60)	7.30±3.64	25.67±10.78	29.08±8.97
Control *GSTM1* null (n=32)	10.18±2.14[a,c]	34.47±5.52 [a,c]	35.50±6.34 [a,c]
Control *GSTM1* positive (n=28)	4.04±1.62[a]	14.86±2.58 [a]	21.39±3.71 [a]

ROS levels (RLU) between *GSTM1* positive and *GSTM1* null genotypes in idiopathic infertile patients and control subjects by One Way ANOVA, Post Hoc Tukey. Data were presented as Mean ±S.D. [a]When compared to patient *GSTM1* null group, p<0.001; [b] When compared to control *GSTM1* positive group, p<0.001; [c] When compared to control *GSTM1* positive group, p<0.001; [d]When compared to control group, p<0.001 (Mann-Whitney U- test).

DISCUSSION

Male subfertility affects 1/10 males and in 30% of cases the origin of reduced male fertility is unknown. It is a heterogeneous disorder, with several genetic and environmental factors contributing to impaired spermatogenesis [19]. Increasing evidence suggests that polymorphisms in several genes are associated with male infertility, although genetic factors that could mediate the pathogenesis of male infertility are mostly unclear. There is also growing evidence to suggest that seminal stress is involved in many aspects of male infertility [20]. Genetic tests have been developed for polymorphisms in several important enzymes that are involved in the protection against oxidative stress. These include polymorphic large deletions causing inactivation of two genes, GSTM1 and GSTT1, that have previously been associated with several conditions where oxidative stress has been implicated [7, 12, 21].

Polymorphism in the genes GSTM1, GSTM3 and GSTM5 have been shown to be associated with male infertility [8, 21, 22]. With respect to GSTM1, it has been suggested that polymorphism of the gene might be an important factor in determining the susceptibility of patients to the development of alcohol-induced disorders of human spermatogenesis [21]. Chen et al. [8] also showed that polymorphism of GSTM1 was related to a susceptibility to infertility in men with varicocele testes. In addition, Okuno et al. [22] showed that the GSTM1 null genotype was associated with a favorable response to varicocelectomy, using an increase in sperm concentration as the outcome. The present study shows an association between GSTM1 gene polymorphism, oxidative stress in spermatozoa from subjects with idiopathic male infertility. Spermatozoa from infertile individuals with the GSTM1 null genotype exhibited greater susceptibility to oxidative stress than that from GSTM1 positive infertile patients. Furthermore, the GSTM1 null genotype was associated with higher ROS, and oxidative stress in the control group. The mean semen sperm concentration was lower in patients with the GSTM1 null genotype than in those possessing the gene.

From epidemiological studies, there is some evidence that genetic variation at the glutathione S-transferase (GST) loci GSTM1 influences individual susceptibility to disease associated with oxidative stress [23]. e The role of the GSTM1 genotype in protection against oxidant chemicals by comparing the cytotoxicity of spermatozoa to low concentrations of BaP- and CumOOH-induced in vitro oxidative challenge be elucidated in future work. Following supplementation with BaP or CumOOH, time-dependent increases were observed in the production of ROS levels after incubation for 10 min. Moreover, time response measurements indicated that increased ROS levels of idiopathic infertile men GSTM1 null genotype were more sensitive to BaP-or CumOOH-induced ROS production in

spermatoza than those with the GSTM1 positive genotype and control subjects GSTM1 null and positive genotypes. These results suggest that spermatozoa from idiopathic infertile men with the GSTM1 null genotype are abnormally susceptible to in vitro induced oxidant challenge, when exposed to CumOOH and BaP. Therefore, the GSTM1 null genotype might predispose spermatozoa of patients with idiopathic infertility to increased oxidative stress. Our findings also suggest that the effect of the GSTM1 genotype might be potentiated in the presence of an additional oxidative burden that might be apparent in idiopathic infertility.

As a result of studies on epithelial ovarian cancer, Sarhanis et al. [11] proposed that GSTM1 might be a critical factor in the detoxification of the products of oxidative stress produced during the repair of the ovarian epithelium. It has also been shown that the GSTM1 enzyme has the highest catalytic efficiency in the detoxication of HAE, which are produced as a result of free radical-initiated lipid peroxidation [24]. Therefore, in individuals with the GSTM1 null genotype, the lack of GSTM1 activity might affect the antioxidant potential within spermatozoa. , The oxidative stress caused by excessive production of ROS has been associated with increased sperm damage and apoptosis [25], and this might account for the lower sperm concentration found in infertile individuals with the GSTM1 null genotype. Further studies are required to investigate the possible contribution of such factors.

In conclusion, the results of the present study suggest that the spermatozoa of idiopathic infertile men with GSTM1 null genotype are more vulnerable to oxidative stress and damage. Therefore, polymorphism of GSTM1 might play an important role in the oxidant/antioxidant systems of spermatozoa in patients with idiopathic male infertility.

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