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Preliminary Examination of Polymorphisms of *GSTM1*, *GSTT1*, and *GSTZ1* in Relation to Semen Quality

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Abstract

Background—Environmental, lifestyle, and occupational exposures on semen quality have been investigated in epidemiological studies with inconsistent results. Genetic factors involved in toxicant activation and detoxification have been examined in relation to the risk of outcomes such as cancer, cardiovascular, and neurologic disorders. However, the effect of common genetic variants in the metabolism of toxicants on semen quality parameters has rarely been evaluated. In this analysis, we evaluated functional SNPs of three genes of the glutathione-S-transferase (GSTM1, GSTT1, GSTZ1) enzyme family.

Methods—Participants were 228 presumed fertile men recruited as part of a community-based study. Semen outcome data from this study included total sperm count and concentration, sperm morphology, and sperm DNA integrity and chromatin maturity. DNA was obtained from 162 men from a mouth-rinse sample and genotyped for the presence of *GSTT1-1* and *GSTM1-1* null genotypes and the *GSTZ1* SNPs at positions 94 (rs3177427) and 124 (rs3177429). We used multivariable linear regression to assess the relationship between each genotype and sperm outcomes.

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Conflict of interest
The authors declare they have no conflict of interest or competing financial interests.

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Results—Overall, our results did not reveal a consistent pattern between *GSTM1* and *GSTZ* genotypes and increased occurrence of adverse sperm outcomes. However, the *GSTT1* non-null genotype yielded the coefficients with the largest magnitude for sperm count and sperm concentration (β= −0.528, 95% CI −1.238-0.199 and β= −0.353, 95% CI −0.708-0.001, respectively), suggesting that it might be adverse.

Conclusions—These results indicate that common polymorphisms in GST genes do not negatively impact sperm parameters in healthy men with good semen quality. Contrary to expectations, the *GSTT1* non-null genotype was associated with reduced sperm concentration and count in semen. Further study with a larger study size and inclusion of gene-exposure interactions is warranted.

Keywords

epidemiology; human; male; reproduction; semen; sperm count; genetic; polymorphism; Glutathione Transferase

1. Introduction

The potential impacts of environmental, lifestyle, and occupational exposures on semen quality have been examined in various epidemiological studies [1–9] Some studies have been limited to routine semen outcomes such as sperm counts, motility, and morphology, whereas more recent studies have also included measures of sperm DNA, and chromosome or chromatin integrity [10–14]. Although mechanistic pathways are rarely examined in these studies, it is suspected that the exposure effects may be mediated through inherited genetic factors involved in toxicant activation and detoxification.

An important enzyme family involved in the detoxification of reactive intermediates is glutathione-S-transferase (GST). These enzymes are involved in the conjugation of reactive intermediates with glutathione, facilitating excretion, and are generally protective. However, GST theta has also been shown to activate some substrates to reactive intermediates that are mutagenic. For example GST theta (GSTT1-1) activates the brominated trihalomethanes, which are disinfection byproducts present in drinking water [15].

Several GST enzymes are encoded by genes with known functional polymorphisms. For example, deletions in *GSTM1* (coding for GST mu1) and *GSTT1* (coding for GST theta1) are relatively common in human populations [16]. Homozygous deletions *GSTM1*0* and *GSTT1*0* result in a lack of enzyme activity [17–18]. These polymorphisms have been associated with an increased risk of cancer, heart disease, and adverse reproductive outcomes [19–21]. In addition, *GSTM1*0* has been examined in relation to semen quality and infertility in a small number of studies [22–24]. GSTZ (coding for GST zeta) catalyzes the glutathione-dependent biotransformation of α-haloacetic acids, including dichloroacetic acid (DCA), a disinfection byproduct, to glyoxylic acid [25]. Promoter and non-synonymous exonic SNPs of the human *GSTZ* have been identified [26–28], resulting in five known isoforms of the GSTZ1 protein. These protein isoforms are a result of four nonsynonomous SNPs (located at the nucleotides positions 23, 94, 124, and 245 corresponding to amino acids 8, 32, 42, and 82). Isoform GSTZ1A contains a Lys rather than a Glu at amino acid 32 and an Arg rather than a Gly at amino acid 42. This isoform has been shown to have increased activity for certain alpha-haloacid substrates, due primarily to an increase in the s resistance to inactivation by dichloroacetate (DCA) [26–27].

We examined the relationship between these common polymorphisms of *GSTM1, GSTT1*, and *GSTZ1* and semen quality, including sperm count, sperm concentration, sperm morphology, and sperm chromatin integrity, among a cohort of presumed fertile men.

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2. Materials and Methods

2.1. Study population

Genotype data were obtained on a subgroup of men who participated in the "Healthy Men Study" (HMS), a study of drinking water disinfection by-products and semen quality. As has been described previously [14, 29], HMS identified male partners of women who participated in a prospective study of drinking water disinfection byproducts and spontaneous abortion and other pregnancy outcomes, the "Right From the Start" study [30–31], conducted in three sites (Raleigh, NC; Memphis, TN; and Galveston, TX). A total of 228 men were included in the primary HMS analysis [14].

2.2. Semen collection and processing

The semen collection and analyses methods have been described in detail elsewhere [14, 29]. Briefly, participants were asked to provide a single semen sample using a special kit designed to allow the man to collect a semen specimen in the privacy of his own home and at a time convenient to him [32]. All samples were packaged with cold packs (necessary to maintain sample stability for the sperm chromatin structure assay, SCSA) and shipped by overnight courier to a single laboratory at the U.S. EPA. Immediately upon receipt, semen volume was measured, and aliquots were removed for determination of sperm concentration by IVOS-IDENT (Hamilton Thorne Research, Beverly, MA.) [33], from which total sperm count was calculated. Additional aliquots were taken to prepare smears that were air-dried and stored for later analyses of sperm morphology [34]. Sperm motility, which declines over time and, therefore, is not a reliable measure for shipped semen, was not included in the statistical analysis. Additional aliquots (0.1 ml) were frozen and stored at −70°C for later analysis of chromatin integrity by SCSA [35], and for chromatin immaturity (protamine deficiency indicated by chromomycin A3 (CMA) staining [36].

2.3. Sperm outcome measures

In this analysis we focused on the following sperm outcomes: sperm count (million/sample) and sperm concentration (million/ml semen), sperm morphology (% normal sperm) including its components (percent of sperm cells with abnormal head, percent of sperm cells with abnormal midsection, percent of sperm cells with abnormal tails, and percent of sperm cells with abnormal cytoplasmic drop), percent sperm with DNA fragmentation, indicative of DNA damage, according to SCSA (%DFI), and % sperm with immature chromatin according to CMA staining.

2.4. Mouth-rinse collection

The present study of genetic polymorphisms was added to the primary HMS project. We re-contacted and recruited approximately 230 male participants in all three study sites who had successfully completed all of the parent HMS study activities (i.e., provided a signed consent form, completed telephone interview, and provided a donation of semen specimen according to study protocol). Men who had declined originally to participate in HMS, dropped out, or failed to complete all study activities were not re-contacted. Once an HMS participant agreed to participate in the polymorphism study, we scheduled the mailing of a mouth-rinse kit for buccal cell collection. At the end of recruitment and follow-up period, 227 recruitment letters were mailed, 188 agreed to participate, and 162 returned their specimen collection kits (response frequency: 162/188 = 86.2%). DNA was isolated from buccal cells by a standard high salt extraction method using Puregene chemistries (Qiagen, Valencia CA).

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2.5. Sample processing and genotyping

A multiplex PCR was performed to analyze for the presence of *GSTT1-1* and *GSTM1-1* [37], and an additional method [38] was also used to confirm the *GSTT1-1* genotype. Genotyping of the *GSTZ1* SNPs at positions 94 (rs3177427) and 124 (rs3177429) was performed on an Applied Biosystems (ABI) 7300 machine using ABI Pre-Designed/Validated TaqMan MGB probes and primers and TaqMan Universal Master Mix (no UNG) and analyzed using ABI software version 2.2. All samples were run in duplicate, and concordance between duplicates was 100%. Out of 162 samples, two could not be genotyped for rs3177427.

2.6. Statistical analyses

Several of the outcome variables were transformed to better approximate the normality assumption of the linear model. Specifically, a natural log transformation was applied to the sperm count and concentration variables, and an arc sine transformation was applied to the percent normal sperm cells, percent of sperm cells with abnormal head, percent of sperm cells with abnormal midsection, percent of sperm cells with abnormal tails, and percent of sperm cells with abnormal cytoplasmic drop. Linear regression was used to assess the association between each genotype variable and each outcome, adjusted for potential confounders (race/ethnicity, age, and study site). Abstinence interval was examined as a potential confounder but was not significant in the model. Although abstinence is related to the semen quality measures, it is not associated with genotype. Thus, it would not be expected to confound the relationship of interest. We also examined history of cigarette smoking (ever/never, amount, and ever/never is the 90- days before semen sample collection), but its adjustment did not materially alter the regression coefficients. For interpretability, each of the outcome variables was standardized (after statistical transformation, if applied) such that the standard deviation and the variance were equal to one. Thus each beta coefficient provides an estimate of effect in terms of a change in standard deviations of the transformed response variable. We used SAS version 9.1 software (Cary, NC) to perform all analyses.

3. Results

The subjects included in the genetic analyses were primarily from Memphis and Raleigh sites (69 and 67, respectively), with only 26 (16%) coming from the Galveston site (Table 1). The majority of the men was white, non-Hispanic, between 25 and 34 years old, and had a college degree. Over 80% of the subjects had household incomes of $40,000 or more. About one-third of the subjects reported smoking cigarettes. Subjects who agreed to participate in the genetic analyses were more likely to be white, non-Hispanic, better educated, and to smoke cigarettes when compared to all of the men eligible for the original HMS study.

Table 2 presents the self-reported racial and ethnic characteristics of the final participants stratified by genotype. None of the participants with the *GSTM1*0* null genotype were Black or Hispanic. Similarly, the *GSTT1*0* null genotype was found only among non-Hispanic participants. None of the heterozygous variant carriers (GA) of the *GSTZ1* SNP 124 were Hispanic. Furthermore, the only carriers of the homozygous variant (AA) of the *GSTZ1* SNP 94 and SNP 124 were white.

Although this study was motivated by reported impacts of GST polymorphisms and metabolism of disinfectant byproducts (mainly brominated trihalomethanes and haloacetic acids), the number of participants was not sufficient to examine interactions with disinfectant byproduct exposures. Therefore, we examined potential associations between the null genotypes and semen outcomes. Overall, our results did not reveal a consistent

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pattern between *GSTM1* and *GSTZ* genotypes and increased occurrence of adverse sperm outcomes. We present the beta coefficients for genotype from the multivariable linear regression, adjusted for age, race, and study site in Table 3. When interpreting the information in Table 3, it is important to note that negative beta coefficients indicate an association between a variant genotype and poorer outcomes for sperm count, sperm concentration and percent normal sperm morphology, whereas positive beta coefficients indicate an association between a variant genotype and elevated DNA/chromatin abnormalities (i.e., percent DFI or CMA).

The comparisons of *GSTT1* genotype with sperm count and sperm concentration yielded the coefficients with the largest magnitude ($\beta = -0.528$, 95% CI −1.238-0.199 and $\beta = -0.353$, 95% CI −0.708-0.001, respectively). This indicated that, on average, men with the *GSTT1*1* non-null genotype (normal levels of the enzyme) would have a sperm count one-half of a standard deviation lower on the log scale and sperm concentration one-third of a standard deviation lower on the log scale than men with the *GSTT1*0* (null) genotype. None of the other comparisons yielded coefficients with higher magnitudes or confidence intervals approaching statistical significance.

4. Discussion

Individuals often respond differentially to environmental exposures, with some exhibiting adverse effects and others not. Variations within genes, including single nucleotide polymorphisms and other small mutations, may account for this differential susceptibility. One of the best studied examples is the family of GSTs, which are multifunctional proteins involved in detoxification of electrophonic xenobiotics [39] and that may act as intracellular transport proteins [40].

The potential importance of GSTs in male reproductive function is implicated by their presence in the testis and seminiferous tubule fluid [41] and on sperm [42], where they are thought to play detoxification and protective roles. The chemoprotective functions of GSTs [reviewed by 43–44] would be expected to be especially important in the testes where GSTs could attenuate the toxicity of reactive oxygen species (ROS) to Leydig cells, Sertoli cells, and germ cells [45]. Developing and maturing sperm are also highly sensitive to lipid peroxidative and DNA damage associated with ROS which, in turn, is associated with male infertility [46–47].

Recently, GST polymorphisms have been examined for their potential to influence susceptibility to damage from reproductive toxicants, including those that impact spermatogenesis and/or damage sperm DNA. The *GSTM1*0* (null) genotype has been found to lower seminal plasma antioxidant capacity, which may result in sperm dysfunction for patients with varicocele [48]. Also *GSST1*1* men (non-null) showed improvement in semen quality after varicocoelectomy (and more so if they were also *GSTM1*1),* whereas men with the null genotype did not [49]. Recently, Messaros et al. [50] reported significantly lower semen quality (numbers, morphology and motility) in men exposed to the organochlorines DDE and DDT, who were also null for *GSTT1* (and carried a polymorphism for *CYP1A1)* than similarly exposed men who carried the common allele for these genes.

These studies suggest that polymorphisms are related to response to toxicants, they may play a more important protective role in infertile than fertile men, and that the ability to respond to oxidative stress depends on multiple genes and enzymes. Two related environmental epidemiology studies found an association between exposure to episodically high levels of industrial air pollution and increased DNA damage in sperm as measured by SCSA [11, 51] A companion study demonstrated a gene-environment interaction whereby men with the

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homozygous deletion of *GSTM1* exhibited an increased susceptibility to the risk of sperm DNA damage (measured with SCSA) associated with this exposure [52].

Here we examined several common genetic variants in three GST genes. The functions of two of these variants have been well characterized. As described above, several previous studies have suggested that the *GSTM1* deletion polymorphism may limit antioxidant potential in reproductive tissue and fluids, thereby increasing oxidative stress and associated adverse effects on semen quality and sperm DNA damage. However, our community-based study of fertile men did not indicate an association, in the absence of an identified exposure, of the *GSTM1* deletion polymorphism or *GSTZ1* SNPs with any of the semen quality measures that we examined, nor with markers of sperm DNA damage. We cannot rule out potential associations with altered sperm motility which is sensitive to oxidative damage, because we were not able to assess sperm motility in our shipped samples. However, in the air pollution study cited above [52] only indicators of DNA damage were associated with high exposures to air pollution in men with *GSTM1* deletions whereas sperm motility and motion parameters were not.

GSTZ1 plays a role in the metabolism of α-haloacetic acids, including several measured in drinking water [25]. We evaluated two GSTZ1 SNPs that have been shown to have functional consequences; however, these variants are rare. For example, only 1 man was a double homozygote for the two GSTZ1 SNPs. Contrary to expectations, the *GSTT1* non-null genotype was associated with lower sperm concentration and count in semen. Our expectation was that the null genotype would confer less detoxification capacity and, thereby, increase the risk of poorer semen quality. We speculate that this apparently contradictory observation may be explained by the ability of the GSTT1 enzyme to operate through an alternate pathway whereby compounds may be activated to reactive intermediates, thus increasing stress, but this was not tested empirically. However, we cannot rule out chance as an explanation for these findings.

This study has several strengths, including that it is a community-based sample of men who are presumed fertile by means of their recruitment through a pregnancy cohort study. This recruitment strategy resulted in a selected population of men who do not have serious underlying reproductive pathologies that could confound the analysis. Indeed, our population had excellent semen quality compared with unselected cohorts or infertility clinics reported in the literature. Also, we were able to examine multiple indicators of semen quality (with the exception of sperm motility) and DNA damage, and we considered data on multiple covariates. In the present analysis, the main effect estimates of most SNPs were relatively precise. This study was not large enough to estimate precisely the interaction between genotype and drinking water disinfectant byproduct exposures. However, disinfectant byproduct exposure was not consistently related to decreased sperm quality in the previous HMS analysis [14].

These results suggest that common polymorphisms in GST genes do not negatively impact sperm parameters in healthy men with good semen quality and in the absence of exogenous oxidative stressors. The lack of association of decreased sperm measures with any single genotype examined here is consistent with the result of Messaros et al. [50] in which the presence of *GSTM1*0* or *GSTT1*0* alone was not associated with decreased semen quality. To the contrary, the *GSTT1*0* genotype is possibly protective in comparison with *GSTT1*1*. Future studies should incorporate a larger study size and include exposures with toxicity pathways related to the enzymes in question in order to evaluate gene-exposure interactions.

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Abbreviations

CMA	chromomycin A3
DCA	dichloroacetic acid
DDE	organochlorine
DDT	organochlorine
GA	heterozygous variant carriers
GST	glutathione-S-transferase
GSTM1	coding for GST mu1
GSTT1	coding for GST theta1
GSTZ	coding for GST zeta
GSTZ1	protein
GSTZ1A	isoform protein
HMS	Healthy Men Study
ROS	reactive oxygen species
SCSA	sperm chromatin structure assay

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Table 1

Selected sociodemographic characteristics for HMS cohorts (based on maternal interview data).

	HMS eligible men (n=274)	HMS final (n=228)	HMS genetic analysis (n=162)
	n (%)	n (%)	n (%)
Site			
Memphis, TN	109 (39.8)	91 (39.9)	69 (42.6)
Raleigh, NC	106 (38.7)	92 (40.4)	67 (41.4)
Galveston, TX	59 (21.5)	45 (19.7)	26 (16.0)
Race/ethnicity[a]			
White, Non-Hispanic	210 (77.2)	187 (82.0)	146 (90.1)
Black, Non-Hispanic	32 (11.8)	18 (7.9)	15 (9.3)
Hispanic	6 (2.2)	0 (0.0)	0 (0.0)
Asian	3 (1.1)	3 (1.3)	0 (0.0)
Other	21 (7.7)	17 (7.5)	1 (0.6)
Paternal age			
<25	38 (14.0)	26 (11.4)	15 (9.3)
25–29	84 (30.9)	71 (31.1)	46 (28.4)
30–34	102 (37.5)	89 (39.0)	71 (43.8)
>=35	48 (17.7)	42 (18.4)	30 (18.5)
Paternal education			
<=High school	56 (20.7)	37 (16.3)	18 (11.1)
Some college	54 (20.0)	42 (18.5)	30 (18.5)
College graduate + some graduate work	160 (59.3)	148 (65.2)	114 (70.4)
Household income			
<=20,000/year	28 (10.4)	19 (8.4)	7 (4.3)
20,001–40,000/year	59 (21.9)	43 (19.1)	22 (13.6)
40,001–80,000/year	125 (46.5)	111 (49.3)	82 (50.6)
>=80,0001/year	57 (21.2)	52 (23.1)	48 (30.2)
Smoke Cigarettes			
No	164(59.9)	136 (59.6)	106 (65.4)
Yes	110(40.1)	92 (40.4)	56 (34.6)

[a]Race/ethnicity for the eligible men (first column) was obtained from the interview with the women. For the final interview, semen and genetic analyses (columns 2 and 3), this information was obtained from the male interview.

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Table 2

Genetic, Racial and Ethnic Characteristics of HMS Participants

	All Sites	Race		Ethnicity				
Covariate	**% (n)**	**% White (n)**	**% Non-White (n)**	**% Asian (n)**	**% Black or African American (n)**	**% Hispanic or Hispanic (n)**	**% White (n)**	**% Other (n)**
GSTM1[a]								
*1	52 (84)	84.5 (71)	15.5 (13)	50 (2)	100 (9)	100 (1)	49 (71)	50 (1)
*0	48 (78)	96.2 (75)	3.8 (3)	50 (2)	0 (0)	0 (0)	51 (75)	50 (1)
GSTT1[a]								
*1	73 (118)	91.5 (108)	8.5 (10)	50 (2)	56 (5)	100 (1)	74 (108)	100 (2)
*0	27 (44)	86.4 (38)	13.6 (6)	50 (2)	44 (4)	0 (0)	26 (38)	0 (0)
GSTZ1_SNP94								
GG	55 (88)	87.5 (77)	12.5 (11)	75 (3)	56 (5)	50 (1)	54 (77)	100 (2)
GA	36 (58)	89.7 (52)	10.3 (6)	25 (1)	44 (4)	50 (1)	36 (52)	0 (0)
AA	9 (14)	100 (14)	0.0 (0)	0 (0)	0 (0)	0 (0)	10 (14)	0 (0)
GSTZ1_SNP124								
GG	90 (145)	88.3 (128)	11.7 (17)	100 (4)	100 (9)	100 (2)	88 (128)	100 (2)
GA	10 (16)	100 (16)	0.0 (0)	0 (0)	0 (0)	0 (0)	11 (16)	0 (0)
AA	1 (1)	100 (1)	0.0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	0 (0)

[a] *1 signifies carrier of gene, *0 signifies homozygous gene deletion

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Table 3

Adjusted[a] Coefficients and 95% Confidence Limits for Sperm Outcomes with Genotype as Exposure Variable

Outcome[b]	GSTM1*0			GSTT1*0			GSTZ1_SNP94 GA or AA			GSTZ1_SNP124 GA or AA		
	Coefficient	LCL[c]	UCL[d]	Coefficient	LCL	UCL	Coefficient	LCL	UCL	Coefficient	LCL	UCL
% Chromomycin A3 (CMA)	−0.033	−0.362	0.296	0.249	−0.109	0.608	−0.122	−0.444	0.201	−0.080	−0.599	0.438
% DNA Fragmentation Index (DFI)	0.042	−0.316	0.400	0.193	−0.186	0.572	0.051	−0.300	0.403	0.062	−0.512	0.636
Sperm Concentration[e]	0.120	−0.206	0.445	−0.353	−0.708	0.001	0.002	−0.312	0.317	0.162	−0.353	0.678
Sperm Count[e]	−0.371	−0.994	0.255	−0.528	−1.238	0.199	0.560	−0.073	1.194	0.174	−0.844	1.182
% Normal Morphology[f]	−0.081	−0.385	0.224	−0.014	−0.352	0.323	−0.125	−0.422	0.171	−0.139	−0.629	0.351

[a] Adjusted for race/ethnicity, age, site

[b] All outcomes standardized such that standard deviation equals variance, which equals 1.00

[c] Lower confidence limit of 95% confidence interval

[d] Upper confidence limit of 95% confidence interval

[e] Natural log transformation applied

[f] Arc Sine transformation applied