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ARTICLE

Synergism between the *N*-acetyltransferase 2 gene and oxidant exposure increases the risk of idiopathic male infertility

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Abstract *N*-acetyltransferase (NAT2) is a phase-II xenobiotic-metabolizing enzyme participating in the detoxification of toxic arylamines, aromatic amines and hydrazines. The present study was designed to investigate whether two common single-nucleotide polymorphisms (SNP) of the *NAT2* gene (481C>T, rs1799929; 590G>A, rs1799930) are associated with susceptibility to idiopathic male infertility and to assess if the risk is modified by oxidant and antioxidant exposures. A total 430 DNA samples (203 infertile patients and 227 fertile men) were genotyped for the polymorphisms by PCR and restriction fragment length polymorphism. No association was found between the *NAT2* polymorphisms and idiopathic male infertility. However, gene–environment interaction analysis revealed that a low-acetylation genotype, 590GA, was significantly associated with increased disease risk in men who had environmental risk factors such as cigarette smoking (OR 1.71, 95% CI 1.02–2.87, $P = 0.042$), alcohol abuse (OR 2.14, 95% CI 1.08–4.27, $P = 0.029$) and low fruit/vegetable intake (OR 1.68, 95% CI 1.01–2.79, $P = 0.04$). This pilot study found, as far as is known for the first time, that the polymorphism 590G>A of *NAT2* is a novel genetic marker for susceptibility to idiopathic male infertility, but the risk is potentiated by exposure to various environmental oxidants.

KEYWORDS: alcohol abuse, cigarette smoking, fruit and vegetable intake, gene–environment interaction, idiopathic male infertility, *N*-acetyltransferase 2 (NAT2)

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Introduction

Male infertility is widespread and affects approximately 10–15% of the global male adult population. Idiopathic infertility (also known as an unexplained infertility) is found in 40–75% of infertile men (Faasse and Niederberger, 2012; Rowe et al., 2000). A progressive increase in prevalence of male infertility and also a decrease in sperm quality have been found worldwide (Carlsen et al., 1992; Jouannet et al., 2001; Kunstmann et al., 1995; Winters and Walsh, 2014). Despite considerable research efforts, the aetiology and the mechanisms underlying male infertility are still the subject of debate (Singh and Jaiswal, 2011). At the same time, a growing body of epidemiological studies clearly suggests that a male infertility epidemic in the world can be related to global changes in the environment over the last decades (Auger et al., 2001; Han et al., 2011; Hansen et al., 2010; Jørgensen et al., 2006). It has been proved that many chemical agents of the environment are responsible for adverse effects on male reproductive organs and spermatogenesis, and are likely to be the leading causes of male infertility in the modern world (Gaspari et al., 2003; Hansen et al., 2010; Horak et al., 2003; Jurewicz et al., 2009; Mendiola et al., 2008).

It is well known that interindividual differences in the ability to activate and detoxify chemical substances of the environment are attributed to the genetic variability of biotransformation or xenobiotic-metabolizing enzymes (Nebert et al., 1996, 2013). Polymorphic genes for xenobiotic-metabolizing enzymes are considered to be important modifiers of susceptibility to male infertility (Aydos et al., 2009; Rubes et al., 2010; Schuppe et al., 2000; Yarosh et al., 2013). Many studies have observed that functional polymorphisms in genes such as *CYP1A1*, *GSTM1* and *GSTT1* are associated with the risk of idiopathic male infertility (Aydos et al., 2009; Jaiswal et al., 2012; Polonikov et al., 2010; Safarinejad et al., 2010).

Arylamine *N*-acetyltransferase 2 (NAT2) is one of the phase-II xenobiotic-metabolizing enzymes that detoxify a number of environment chemicals such as arylamines, hydrazines, aromatic and heterocyclic amines into their intermediates through reactions of *N*-acetylation and *O*-acetylation (Blum et al., 1991; Hein et al., 2000). Human *NAT2* is expressed in the male reproduction system, including testicular tissues, prostate, genital ducts and exocrine glands where the enzyme may have a protective role against chemicals responsible for male urogenital diseases (Husain et al., 2007; Wu et al., 2013). In particular, the enzyme acetylates benzidine and 2-naphthylamine (harmful aromatic amines of cigarette smoke), 2-aminofluoren (a dye widely used in industry), hydrazine-containing drugs (isoniazid, simendan) and heterocyclic amines (chemicals in meat cooked at high temperatures) are derived from their metabolic activation by cytochrome P450 1A2 (Guengerich, 2000; Hein, 2002; Hein et al., 2000). NAT2 has also been found to activate carcinogens, thereby producing unstable electrophiles that may induce point mutations in DNA (Hein et al., 1993).

NAT2 is a highly polymorphic intronless gene having more than 60 alleles (Vatsis et al., 1995). Polymorphisms in *NAT2* are responsible for the slow (homozygous carriers for low-activity alleles) and rapid (carriers of one or more high-activity alleles) acetylator phenotypes, each occurring with a frequency of about 50% in European and African populations and slightly less frequently in other racial/ethnical groups of the world (Hein et al., 2000). The SNP 481C>T (rs1799929) and 590G>A (rs1799930) are located and are known to be the most common and functionally important genetic variations in *NAT2* (Hein, 2002; Hein et al., 2000). It has been found that a C > T nucleotide substitution at position 481 does not alter the amino acid chain of the enzyme (known as a synonymous SNP, L161L), whereas a G > A substitution at nucleotide 590 causes an amino acid change from arginine to glutamine at codon 197 (R197Q; Cascorbi et al., 1995). These polymorphisms are in linkage disequilibrium and result in decreased activity of NAT2 enzyme, determining a low acetylator phenotype in men (Cascorbi et al., 1995).

Acetylator phenotype and related genetic variants of *NAT2* have been associated with the risk of various types of cancers (Cui et al., 2011; Gong et al., 2011a; Ma et al., 2013; Ying et al., 2011; Zheng et al., 2012; Zhong et al., 2010). In recent years, much attention of andrologists has been given to investigating the roles of *NAT2* in the development of cancers affecting the male reproductive system (Agúndez, 2008; Gong et al., 2011b). As far as is known, no studies have been designed to investigate the associations between *NAT2* polymorphisms and the risk of idiopathic infertility in men.

The purpose of this pilot study was to investigate whether two common SNP of *NAT2*, 481C>T and 590G>A, are associated with susceptibility to idiopathic male infertility. It was also important to assess whether oxidant and antioxidant exposures of the environment modify the relationship between *NAT2* polymorphisms and disease risk.

Materials and methods

Study population and diagnosis

The study was performed in keeping with the principles of the Helsinki Declaration. Each participant gave informed consent before enrollment in the study. The study protocol was approved by the Ethical Review Committee of Kursk State Medical University (reference number 6–07, approved 4 October 2007).

A total 430 unrelated Russian men were recruited for the study from the Family Planning and Reproductive Health Clinic of Kursk Regional Perinatal Centre over a period from 2006 to 2008. All eligible patients and controls matching the inclusion/exclusion criteria were given the opportunity to be enrolled in the study groups. The case group comprised 203 men with diagnosis of idiopathic male infertility. Criteria for inclusion in the case group were infertility for at least 12 months of regular unprotected intercourse with at least two repeated findings of abnormal sperm parameters and negative mixed agglutination reaction test. All recognizable causes of male infertility (varicocele, hypogonadotrophic hypogonadism, abnormal karyotypes, microdeletions of chromosome Y, abnormal sexual and ejaculatory functions and seminal tract obstruction) were excluded by a experienced andrologist, endocrinologist, geneticist and laboratory assistants. Only males with normal female partners of ovulatory age were included in the study; female infertility factor was excluded by experienced gynaecologists after thorough instrumental and laboratory investigations, including hysterosalpingography and/or hysteroscopy, biochemical tests for serum hormone profiles, karyotyping and molecular analysis for urogenital infections. The control group included 227

healthy fertile volunteers who had fathered at least one child and had normal semen parameters according to World Health Organization guidelines (Rowe et al., 2000).

Semen specimens were taken by masturbation after at least 2 days of sexual abstinence. All fertile volunteers underwent standard andrological examination in order to exclude the possibility of urogenital disorders. Possible factors confounding the risk of male infertility, such as smoking status, alcohol habit, medication use, body mass index, educational level and occupation, were not used as matching criteria for the case and control groups during patient recruitment. The age was higher in the case group than in the controls (34.4 years versus 29.2 years, $P < 0.01$).

Assessment of oxidant and antioxidant exposures

All study participants completed an interviewer-administered questionnaire concerning demographic data, cigarette smoking habit (ever or never smokers), alcohol consumption and intake of fruits and vegetables. A quantitative alcohol consumption questionnaire was earlier described (Polonikov et al., 2011). Responders were categorized into the following subgroups according to alcohol drinking habit: (i) alcohol abusers (62 patients and 75 controls) who consumed alcohol 3 or more days per week; and (ii) low and moderate alcohol consumers (141 patients and 105 controls) who consumed alcohol once a month or less often and 1–2 days per week, respectively. Fruit and vegetable intake was evaluated by a dietary assessment screener as described previously (Polonikov et al., 2009a). Responders who consumed fruits and vegetables two or more times per day are considered to be high fruit/vegetable consumers (32 patients and 20 controls) and low fruit/vegetable consumers were defined as those who consumed fruits and vegetables once a day or less often (134 patients and 113 controls).

DNA extraction and genotyping

Genomic DNA was purified from whole-blood samples through phenol/chloroform extraction and ethanol precipitation according to standard procedures. The selection of the SNP for this study was based on their high allele frequencies in the population and significant impact on activity of the NAT2 enzyme (Hein, 2002; Hein et al., 2000). SNP were genotyped by PCR followed by restriction fragment length polymorphism analysis according to the protocols described elsewhere (Hickman et al., 1992). PCR amplification with the primer pair 5′-GCTGGGTCTGGAAGCTCCTC-3′ (forward) and 5′-TTGGGTGATACATACACAAGGG-3′ (reverse) generated a *NAT2*-specific 547-bp fragment. PCR was performed in a final reaction volume of 25 μl containing 1.5U of a thermostable Taq DNA polymerase (Lytech, Moscow, Russia), ~ 1 μg DNA, 0.25 μmol/l each primer, 250 μmol/l dNTP, 2.5 mmol/l $MgCl_2$ and ×1 PCR buffer (67 mmol/l Tris HCl, pH 8.8, 16.6 mmol/l $(NH_4)_2SO_4$, 0.01% Tween 20). PCR was run using an annealing temperature of 60°C on thermal cycler (DNA-Technology, Moscow, Russia). PCR products were incubated with 5 U *Kpn*I (SNP 481C>T) and *Taq*I (SNP 590G>A) overnight (Sibenzyme, Novosibirsk, Russia). The digested DNA fragments were separated on 3% ethidium-bromide-stained agarose gels and visualized under ultraviolet light using a GDS-8000 Computer Detection System (UVP, Upland, USA). Genotyping was blinded to the case/control status and the repeatability test was conducted for about 10% of the samples, resulting in a 100% concordance rate.

Statistical analysis

Statistical power was calculated for a chi-squared test using a two-sided *P*-value of 0.05 based on frequencies of the *NAT2* genotypes in the study groups. Power calculations were based on the *NAT2* allele and genotype frequencies previously investigated in Kursk population (Polonikov et al., 2009b). This work was able to detect a difference of 10–18% between groups with power of 75% and a 5% type I error on the basis of sample sizes for each subgroup analysis.

Allele frequencies of the *NAT2* polymorphisms were estimated by the gene counting method. The chi-squared test was used to identify departures from Hardy–Weinberg equilibrium. The allele and genotype frequencies between the groups were compared by chi-squared analyses for 2 × 2 contingency tables. Logistic regression analyses were performed to examine the associations of the *NAT2* genotypes with male infertility risk. Odds ratios (OR) with their 95% confidence intervals (CI) were calculated to estimate a magnitude of the association between the *NAT2* genotype and the disease risk. Haplotype frequencies for the polymorphisms were calculated using the Estimation of Haplotypes program (Xie and Ott, 1993). Pairwise nonstandardized linkage disequilibrium coefficients (*D*) for each SNP were estimated by the maximum-likelihood method from the frequencies of genotypes in 3 × 3 contingency tables under an assumption of the codominance of loci. Gene–environment interactions were analysed by joint categories of the *NAT2* genotype and environmental factors with respect to male infertility risk using binary logistic regression. A two-sided *P*-value <0.05 was considered to be statistically significant. STATISTICA for Windows version 8.0 (StatSoft, Tulsa, USA) was used for all calculations.

Results

Table 1 shows the distribution of alleles and genotypes for polymorphisms 481C>T and 590G>A of *NAT2* in infertile and fertile men. The allele and genotype frequencies of the *NAT2* polymorphisms in each study group satisfied the Hardy–Weinberg equilibrium law. The frequencies of alleles and genotypes of both SNP were comparable to those previously reported in Kursk population (Polonikov et al., 2009b). As can be seen from **Table 1**, no significant difference in allele and genotype frequencies of *NAT2* were found between idiopathic male infertility patients and healthy men.

Table 2 shows the haplotype frequencies for the *NAT2* polymorphisms in the study groups. Four *NAT2* haplotypes were estimated. Haplotypes H1, H2 and H3 were found to be most common (each with a frequency more than 25%), whereas the frequency of haplotype H4 was <1% in both groups. There were no statistically significant differences in the distribution of the *NAT2* haplotypes between the cases and controls. The

Table 1 Distribution of alleles and genotypes of *NAT2* polymorphisms among infertile and fertile men.

NAT2 *polymorphism*	*Infertile men* (n = *203)*	*Fertile men* (n = *227)*	*Chi-squared*
481C>T (rs1799929)			
Alleles			
C	235 (57.9)	274 (60.4)	0.54
T	171 (42.1)	180 (39.6)	
Genotypes			
CC	70 (34.5)	79 (34.8)	0.00
CT	95 (46.8)	116 (51.1)	0.79
TT	38 (18.7)	32 (14.1)	1.68
590G>A (rs1799930)			
Alleles			
G	294 (72.4)	338 (74.4)	0.46
A	112 (27.6)	116 (25.6)	
Genotypes			
GG	103 (50.7)	128 (56.4)	1.38
GA	88 (43.3)	82 (36.1)	2.34
AA	12 (5.9)	17 (7.5)	0.42

Values are *n* (%). No statistically significant differences were found.

Table 2 Distribution of haplotype for *NAT2* polymorphisms in infertile and fertile men.

Designation	NAT2 *haplotype*	*Infertile men* (n = *203)*	*Fertile men* (n = *227)*	*Chi-squared*
H1	481C/590G	0.314259	0.348025	5.52
H2	481C/590A	0.264558	0.255500	
H3	481T/590G	0.409879	0.396469	
H4	481T/590A	0.011304	0.000007	

No statistically significant differences were found.

Table 3 Distribution of environmental risk factors in infertile and fertile men.

Environmental risk factor	*Infertile men*	*Fertile men*	*Chi-squared (P-value)*
Smoking	*n* = 203	*n* = 194	
Smokers	135 (66.5)	116 (59.8)	1.92 (NS)
Non-smokers	68 (33.5)	78 (40.2)	
Alcohol drinking	*n* = 203	*n* = 180	
Alcohol abusers	62 (30.5)	75 (41.7)	5.14 (0.02)
Low or moderate alcohol consumers	141 (69.5)	105 (58.3)	
Fruit/vegetable intake	*n* = 166	*n* = 133	
Low fruit and vegetable consumers	134 (80.7)	113 (85.0)	0.92 (NS)
High fruit and vegetable consumers	32 (19.3)	20 (15.0)	

Values are *n* (%). Degree of freedom = 1. NS = not statistically significant.

polymorphisms 481C>T and 590G>A were in negative linkage disequilibrium in infertile patients ($D = -0.105$, $P < 0.0001$) and healthy controls ($D = -0.101$, $P < 0.0001$).

We recently proposed that analysis for gene–environment interactions might be a fruitful method in unravelling the mechanisms by which the candidate gene is involved in the pathogenesis of male infertility (Yarosh et al., 2013). The present study tested the hypothesis whether the *NAT2* polymorphisms influence the risk of male infertility depending on oxidant and antioxidant exposures. For this purpose, cigarette smoking status and alcohol abuse in contrast to a high intake of fruits and vegetables were chosen as surrogate factors of the environment representing oxidant and antioxidant exposures at the individual level, respectively. The distribution of these factors in the study groups is shown in Table 3. Both groups were comparable with respect to smoking status. Data on fruit and vegetable intake were available from 166 infertile and 133 fertile men. There were 134 (80.7%) of 166 idiopathic male infertility patients and 113 (85.0%) of 133 healthy men who were low fruit/vegetable consumers (once a day or less often). This means that there was no difference in the rate of fruit and vegetable intake between the study groups (chi-squared = 0.92). A surprising finding was that the rate of alcohol abusers in fertile men was higher then

Table 4 Gene–environment interaction analysis and susceptibility to idiopathic male infertility.

NAT2 *genotype*	*Infertile men* (n = *203)*		*Fertile men* (n = *227)*		*OR (95% CI)*[a]	
	With risk factor	*Without risk factor*	*With risk factor*	*Without risk factor*	*With risk factor*	*Without risk factor*
Cigarette smoking						
590GG	67 (49.6)	35 (51.5)	71 (61.2)	39 (50.0)	1.60 (0.97–2.65)	0.94 (0.49–1.81)
590GA	60 (44.4)	29 (42.6)	37 (31.9)	34 (43.6)	**1.71 (1.02–2.87)**	0.96 (0.50–1.86)
590AA	8 (5.9)	4 (5.9)	8 (6.9)	5 (6.4)	0.85 (0.31–2.34)	0.93 (0.26–3.39)
Alcohol abuse						
590GG	29 (46.8)	74 (52.5)	49 (65.3)	56 (53.3)	**2.14 (1.08–4.27)**	1.03 (0.62–1.72)
590GA/AA	33 (53.2)	67 (47.3)	26 (34.7)	49 (46.7)		
Low fruit/vegetable intake						
590GG	61 (45.5)	16 (50.0)	66 (58.4)	13 (65.0)	**1.68 (1.01–2.79)**	1.80 (0.59–5.54)
590GA/AA	73 (54.5)	16 (50.0)	47 (41.8)	7 (35.0)		

Values are *n* (%). Degree of freedom = 1. Statistically significant associations are indicated by boldface.
[a]Odds ratio (95% confidence intervals), *df* = 1.

in patients with idiopathic infertility (chi-squared = 5.14, $P = 0.02$).

Table 4 shows the results of the gene–environment interaction analysis for polymorphism 590G>A. Although associations between 590G>A and idiopathic male infertility risk were not found in the preliminary analysis, joint effects of this polymorphism and each environmental factor on the disease risk were observed. In particular, the heterozygous genotype 590GA was associated with an increased risk of idiopathic infertility in smokers (OR 1.71, 95% CI 1.02–2.87, $P = 0.042$), whereas nonsmokers possessing this genotype did not have disease risk (OR 0.96, 95% CI 0.50–1.86). Men with the variant genotypes 590GA and 590AA were at an increased risk of infertility if they were alcohol abusers (OR 2.14, 95% CI 1.08–4.27, $P = 0.029$). Furthermore, carriers of genotypes 590GA and 590AA had an increased risk of developing male infertility if they were low fruit and vegetable consumers (OR 1.68, 95% CI 1.01–2.79, $P = 0.04$). Meanwhile, the carriers of these genotypes who were high fruit and vegetable consumers did not have a risk of male infertility (OR 1.80, 95% CI 0.59–5.54). The polymorphism 481C>T did not show an association with male infertility risk in the analysis stratified by the environmental factors (data not shown).

Discussion

In the gene coding for arylamine *N*-acetyltransferase 2, several polymorphisms have been identified, resulting in enzymes with increased or reduced enzyme activity and determining whether people are rapid, intermediate or slow acetylators (Blum et al., 1991). *NAT2* polymorphisms have been associated with an increased risk of male malignancies, such as urinary bladder (Green et al., 2000) and prostate cancer (Hein et al., 2002). Importantly, these types of cancers are known to be induced by certain environmental carcinogens which are metabolized by NAT2 enzyme (Carreón et al., 2006; Costa et al., 2005; Gong et al., 2011a; Su et al., 1998). Oxidative stress is a well-known mechanism of chemical- and/or carcinogen-induced tumours, resulting from an imbalance between the production of reactive oxygen species (ROS) and their efficient removal by available antioxidant defence systems (Valko et al., 2006). Notably, oxidative stress together with associated sperm DNA damage and apoptosis have been clearly implicated in the pathogenesis of idiopathic infertility in men (Agarwal and Said, 2005; Aitken et al., 2003; Gharagozloo and Aitken, 2011; Sanocka et al., 1997). Since oxidative stress is thought to be involved in the pathogenesis of idiopathic male infertility, polymorphisms of *NAT2* may represent potential candidates for disease susceptibility.

The aim of this pilot study was to investigate the association of two common polymorphisms of *NAT2* with the risk of idiopathic infertility and to elucidate a trigger role of cigarette smoking, alcohol abuse and low fruit and vegetable intake in the development of the disease depending on the carriage of the *NAT2* genotypes. Despite the *NAT2* genotypes not showing an association with idiopathic male infertility risk in an initial analysis, subsequent analysis for gene–environment interactions showed that the genotype 590GA was associated with male infertility in men who were smokers, abused alcohol or had low fruit/vegetable diets; as far as is known these results have been obtained for the first time. These findings suggest that environmental exposures characterized by opposite effects on the redox homeostasis (i.e. pro-oxidant and antioxidant exposures) may modify the relationship between the *NAT2* genotype and male infertility risk.

It is well known from the literature that oxidative stress is considered to be the major pathological mechanism of tissue injury of the male reproductive tract occurring during cigarette smoking (de Jong et al., 2014; Zenzes, 2000) and excessive alcohol drinking (de Jong et al., 2014; Emanuele and Emanuele, 2001a). Allele 590A is part of the *NAT2*6A* haplotype, which causes the slow-acetylator phenotype due to producing proteins that are poorly expressed, unstable or possess reduced catalytic activities (Badawi et al., 1995). It can be assumed that alcohol abuse and chemical toxicants of tobacco smoke in the carriers of low-acetylator genotype 590GA are responsible for higher production of ROS, thereby enhancing susceptibility to infertility. Moreover, low fruit/vegetable intake was found to be associated with the increased risk of infertility only in carriers of this genotype. It can be proposed that a low fruit/vegetable diet seems to

enhance oxidative stress through weakening of the antioxidant defence mechanisms in carriers of the *NAT2* genotype. The harmful effects of such environmental factors on the male reproductive system have been demonstrated by several epidemiological studies. In particular, cigarette smoking has been found to negatively influence sperm concentration, morphology and motility (Künzle et al., 2003; Meri et al., 2013) possibly through inducing seminal oxidative stress (de Jong et al., 2014; Saleh et al., 2002). Alcohol can induce oxidative damage either by enhancing the production of highly toxic ROS from alcohol and acetaldehyde, producing lipid peroxidation of membranes in testicular cells or decreasing the concentrations of antioxidants (Emanuele et al., 2001b). Ethanol has also been found to suppress testicular testosterone production and release (Gianoulakis, 1990) and increase apoptosis of Leydig and seminiferous cells (Yin et al., 1999), ultimately affecting sperm parameters (Sermondade et al., 2010) and decreasing fertilization rate (Braga et al., 2012). Undoubtedly, the influence of alcohol intake on the seminal quality has dose-effect and time-effect relationships and depends on the activity of ethanol-metabolizing enzymes.

Moreover, tobacco smoke contains toxic aromatic amines (benzidine and 2-naphthylamine) which are substrates for *N*-acetyltransferase-2 (Hein et al., 2002). These chemicals are known to be associated with increased risk of diseases of the male urogenital tract, including urinary bladder neoplasms (Carreón et al., 2006; Cohen et al., 2000) and prostate cancer (Carreón et al., 2006; Srivastava and Mittal, 2005). A possible explanation of the relationship between tobacco smoking and male infertility risk at a diminished activity of NAT2 is that increased concentrations of tobacco-related toxicants such as benzidine and 2-naphthylamine may be responsible for the induction of oxidative stress in male reproductive organs.

It is important to add that the NAT2 enzyme is also involved in the metabolism of commonly used drugs that are known to affect the male reproductive system and be responsible for chemically induced abnormalities of spermatogenesis. In particular, sulphasalazine and sulphonamide drugs, substrates for NAT2, are able to depress sperm motility and acrosome reaction by means of increasing oxidative stress (Alonso et al., 2009; Fukushima et al., 2005).

In contrast, a higher consumption of fruits and vegetables has a beneficial effect on male infertility, as it has been noted in some recent studies (Braga et al., 2012; Eslamian et al., 2012a). Natural antioxidants which are present in fresh fruits and vegetables seem to ameliorate sperm parameters, thereby explaining their protective effects on male infertility risk (Eslamian et al., 2012b; Mínguez-Alarcón et al., 2012).

The limitations of this study need to be considered. First, it should be noted that association analyses stratified by environmental exposures were based on a limited number of men in each study group, lowering the power of the analyses. This was a reason why the joint categories of all environmental factors and the *NAT2* genotypes were not evaluated simultaneously in association analyses. Since infertile men may have a number of risk factors, it is pretty difficult to investigate the effects of a *NAT2* polymorphism free of other confounding variables known to be related with disease risk. Moreover, the questionnaire on fruit and vegetable intake used in this study was based only on the frequency question and did not assess quantitative and qualitative characteristics of the intake. Second, due to the observational nature of this study, the findings should be confirmed in larger studies in order to replicate the association of *NAT2* polymorphisms with idiopathic infertility and its dependence on the environmental exposures. Third, this work cannot exclude the possibility that the polymorphism 590G>A is a marker for other functional variants of *NAT2*. In this connection, further studies should be focused not only on the investigations of a much broader spectrum of SNP (for example, tag SNP) in *NAT2* but also on their functional significance through the analysis of gene expression and/or enzyme activity.

The present study is the first, as far as is known, to report that *N*-acetyltransferase type 2 is a novel candidate gene for susceptibility to idiopathic infertility in men. The findings demonstrate that *NAT2* gene–environment interactions may play an important role in the pathogenesis of idiopathic male infertility and that the analysis of joint effects of genetic and environmental factors may provide clues to the molecular mechanisms of the disease. Notably, the association between *NAT2* and male infertility is modified by oxidative stress related environmental factors such as cigarette smoking, alcohol abuse and low fruit/vegetable intake, which are known to influence the risk of the disease. Taken together, the study results support the hypothesis for toxicogenetic nature of idiopathic male infertility and provide novel insights into molecular mechanisms of the disease.

From a practical point of view, these results underscore recommendations for the arrest of cigarette smoking and alcohol abuse and for encouraging the consumption of diets rich in fruits and vegetables as a means of male infertility prevention in reproduction medicine. Useful medical applications of these results can be also realized through the clinical evaluation of antioxidant therapy in infertile men to effectively manage sperm oxidative stress and ultimately to ameliorate this condition, as has been recommended (Lanzafame et al., 2009). From a research perspective, a pathway-based approach where several genes for xenobiotic-metabolizing and antioxidant defence enzymes are selected for analysis in combination with the knowledge of the chemical factor can represent a more comprehensive and fruitful strategy to identifying the joint effects and gene–environment interactions underlying male infertility.

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