Correlation analysis of age and *MTHFR* C677T polymorphism with sperm motility and sperm DNA integrity

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ABSTRACT

Several factors, including age and polymorphisms in genes such as *MTHFR*, affect sperm quality. However, the relationship between MTHFR C677T polymorphism and infertility remains unclear. Hence, the purpose of this study was to investigate the effects of age and methylenetetrahydrofolate reductase (*MTHFR*) C677T polymorphism on the forward progressive sperm motility and sperm DNA integrity. For this purpose, a total of 845 men were recruited from the hospital's infertility clinic for semen analysis, sperm DNA integrity testing, and MTHFR C677T genotyping. The participants were grouped by age (<30 years, 30−35 years, >35 years) to determine the relationship between age and the progressive motility of sperms (PR%) and sperm DNA integrity. The effect of MTHFR C677T polymorphism on sperm motility and sperm DNA integrity was also analyzed in all the age groups. Results showed that PR% and DNA fragmentation index (DFI) differed significantly among the groups ($P<0.001$). The correlation analysis showed that age was positively correlated with DFI and negatively correlated with PR%. DFI correlated negatively with PR% ($P <0.01$). When the age groups were classified based on the C677T genotype, we observed that PR% and DFI did not differ significantly between different genotypes within the same age range ($P>0.05$). The conclusion was that age exerted a negative effect on sperm motility and sperm DNA integrity. Age correlated positively with DFI and negatively with PR%. *MTHFR* C677T polymorphism did not affect forward motile sperm count and sperm DNA integrity. Our observations will be useful for fertility guidance and *MTHFR* genotype interpretation in the clinic for couples of childbearing age.

Introduction

Infertility is a global health problem; infertile couples of childbearing age account for 12−15% of the population, and male and female factors each are responsible for approximately 50% of these cases (1,2). Factors affecting male infertility are complex and diverse, and problems may occur in any step of spermatogenesis and sperm maturation to cause male infertility (3). Considering the current state of fertility worldwide, laboratory tests for evaluating male fertility are being improved, including basic tests for determining sperm concentration, motility, morphology, DNA integrity, seminal plasma biochemistry, and genetic testing for specific problems.

Some recent studies have investigated the influence of gene polymorphisms on male fertility. Methylenetetrahydrofolate reductase (MTHFR) is an important flavin-dependent enzyme involved in the metabolism of folic acid and homocysteine (Hcy); it contributes to DNA synthesis, repair, and methylation by mediating the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate (4). *MTHFR,* located on 1p36.3, is 20.374 kb long and contains 12 exons. C677T is the most common *MTHFR* polymorphism located on exon 4. The C677T mutation results in 35% decrease in MTHFR activity in heterozygotes and 70% decrease in homozygotes. The resulting hyperhomocysteinemia may damage sperm DNA (5). However, the relationship between *MTHFR* C677T polymorphism and infertility remains unclear. A meta-analysis showed that *MTHFR* polymorphisms, especially the C677T subtype, were associated with male infertility (6).

Infertile men have higher levels of DNA fragmentation than fertile men, and sperm DNA damage is strongly associated with male infertility (7,8). The importance of the sperm DNA fragmentation (SDF) assay has also been recognized in the latest American Urological Association (AUA)and European Association of Urology guidelines on male infertility (9). The progressive motility of sperm (PR%) is an important indicator of sperm motility. Age is an important factor affecting male fertility, and aging shows a significant negative correlation with sperm DNA integrity and other sperm parameters (10). Hence, in this study, we stratified the participants according to age and then investigated the effect of *MTHFR* polymorphism on sperm DNA integrity and motility. We believe that our observations regarding the relationship between age and *MTHFR* polymorphism with sperm quality will guide clinical reproductive therapy.

Materials and Methods

Subjects

This was a retrospective study. The subjects were 845 male patients who visited the infertility clinic of the Re-

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productive Medicine Center of the Xinjiang Medical University Affiliated First Hospital, China, from July 2021 to January 2022. The inclusion criteria were as follows: (I) age, 20 ~ 60 years; (II) no abnormalities on examination of the male reproductive system, including male secondary sexual characteristics, penis, scrotum, spermatic cord, vas deferens, epididymis, and testis; (III) abstinence from sex for 2 to 7 days before semen collection (11); (IV) semen samples collected after masturbation in a dry and sterile sperm collection cup and submitted immediately for an examination. Cases of azoospermia and severe oligozoospermia (sperm concentration $<5 \times 10^6$/mL) were excluded from the study.

Semen analysis

Using a completely automated sperm analyzer (BEIONS6), five fields of view were randomly selected under an optical microscope to count the total number of sperms and forward-moving sperms. PR% was calculated as rapid progressive motility sperm/total sperm × 100%.

Sperm DNA integrity

Sperm DNA integrity was detected using a sperm chromatin dispersion (SCD) kit (Anhui Anke Bioengineering Company). Briefly, 15 μL semen sample was added to a tube with flow agarose and blended gently. Then, 25 μL of the mixture was deposited on a pretreated slide and covered with a glass coverslip. The slides were removed after 10 min in a 4°C refrigerator and rinsed with distilled water, following which the coverslip was removed. This was followed by 6 min of acid denaturation and 25 min of lysis to remove nuclear proteins. Next, the slides were allowed to stand in distilled water for 5 min, and dehydrated successively in 70%, 90%, and 100% ethanol baths for 3 min each. After Wright staining, the number of sperms with large halo rings (halo ring thickness $>2/3^{rd}$ the minimum diameter of sperm head), medium halo rings ($1/3^{rd}$ the minimum diameter of sperm head < halo ring thickness $\leq 2/3^{rd}$ the minimum diameter of sperm head), small halo rings (halo ring thickness $\leq 1/3^{rd}$ the minimum diameter of sperm head), and no halo rings, and degraded sperms were counted under the microscope (Olympus CX21). Sperm DNA fragmentation index (DFI) (%) = (small halo ring sperm + no halo ring sperm + degraded sperm)/total sperm count × 100%.

Detection of the *MTHFR* C677T genotype

For detecting the *MTHFR* C677T genotype, 2 μL of venous blood was collected and anticoagulated with EDTA. DNA was extracted from whole blood and the gene was amplified using polymerase chain reaction (PCR) on a real-time quantitative PCR detector (Roche Light Cycler480); The ABI9700 PCR instrument was used for sequence analysis and NanoDrop 2000 was used to determine DNA concentration (Thermo Scientific). The nucleic acid extraction kit and *MTHFR* C677T genotype detection kit were from Shanghai Bioao Technology Company. The amplification program was as follows: 50°C for 5 min, 94°C for 5 min (one cycle); then, 35 cycles of 94°C for 25 s, 56°C for 25 s, 72°C for 25 s; 72°C for 5 min. The sample genotype was determined using the chip hybridization color.

All the above tests were performed in the First Affiliated Hospital of Xinjiang Medical University (the equipment used in the experiment was located in the First Affiliated Hospital of Xinjiang Medical University)

Grouping criteria

The patients were grouped by age as follows: <30 years, 30 to 35 years (including 30 and 35 years), and >35 years old. Based on the *MTHFR* C677T genotyping results, the patients were divided into CC (wild type), CT (heterozygous), and TT (homozygous mutant) groups. For analyzing the differences in PR% with respect to the *MTHFR* C677T genotype, PR% values were grouped as PR% $\geq$ 32%, 10% $\leq$ PR% < 32%, 1% $\leq$ PR% < 10%, PR% < 1% (12,13).

Statistical analysis

SPSS 26.0 was used for statistical analysis. Quantitative data were expressed as mean ± standard deviation (x ± s) and enumeration data were expressed as rate (%). Quantitative data conforming to normal distribution were first subjected to oblique analysis of variance to remove confounding factors and then multi-group analysis of variance, and enumeration data were compared using the χ^2 test, Correlation between multiple variables was determined using Pearson correlation analysis. $P <0.05$ was considered statistically significant.

Results

In total, 845 patients were included in the study. The PR% and DFI obtained after grouping by age is shown in Table 1. The <30 years group included 162 cases, accounting for 19.2% of cases; PR% was 56.38 ± 15.69 and DFI was 16.28 ± 8.14 for this group. The 30~35-year-old group comprised 461 cases, accounting for 54.6% of the cases, the PR% of which was 52.29 ± 16.27 and DFI was 17.98 ± 9.29. The >35-year-old group comprised 222 cases, accounting for 26.3% of the cases, with PR% of 48.81 ± 17.74 and DFI of 20.46 ± 10.39. The differences in PR% and DFI among the age groups were statistically significant ($P<0.001$).

Further, correlation analysis (Table 2) revealed that age correlated negatively with PR%. Age and DFI correlated positively; DFI correlated negatively with PR%.

The distribution of the genotypes of *MTHFR* C677T were as follows: wild type (CC), 253 cases (29.9%); hete-

Table 1. Results of PR% and DFI grouped by age.

Age (years)	N (%)	PR%	DFI
<30	162 (19.2)	56.38±15.69	16.28±8.14
30~35	461 (54.6)	52.29±16.27	17.98±9.29
>35	222 (26.3)	48.81±17.74	20.46±10.39
F		9.921	9.825
P		<0.001	<0.001

PR%, progressive motility; DFI, DNA fragmentation index.

Table 2. Correlation analysis of age, PR%, and DFI.

	Age	PR%	DFI
Age	1		
PR%	-0.108**	1	
DFI	0.194**	-0.525**	1

Note: **: *P*<0.01. PR%, progressive motility; DFI, DNA fragmentation index.

rozygous genotype (CT), 376 cases (44.5%); homozygous mutant genotype (TT), 216 cases (25.6%). The results of the *MTHFR* C677T locus test were in agreement with Hardy-Weinberg genetic equilibrium.

The various ranges of PR% in the *MTHFR* C677T genotypes were compared in the different age groups (Table 3). PR% did not differ significantly among the different genotypes in the three age groups.

DFI among the *MTHFR* C677T genotypes in the same age interval was compared (Table 4). We did not find any significant difference in DFI among the different genotypes in the three age groups.

Discussion

The study aimed to establish the effect of age on sperm motility and sperm DNA integrity. We also investigated the effect of *MTHFR* C677T polymorphism on forward sperm count and sperm DNA integrity.

Relationship between age, PR%, and DFI
The results of this study showed that PR% decreased with age; the older the age, the lower the PR%. DFI increased with age; the older the age, the higher the DFI. These results are consistent with those of other studies, that indicated age is a critical factor for male fertility (14-17).

Age affects sperm motility and sperm DNA integrity via the following mechanisms:

Age-related overproduction of reactive oxygen species (ROS), limited antioxidant defense of sperm, and oxidative stress-induced sperm damage. Studies have shown that sperm nuclear DNA fragmentation correlates posi-

tively with ROS overproduction (18,19). The imbalance between ROS and antioxidant levels can also lead to the oxidation of fatty acids in the sperm membrane, leading to changes in sperm motility (20).

Male aging is often associated with DNA repair in defective sperms, which can lead to abnormal chromatin packaging and reduced ability to repair broken DNA strands (21,22).

Aging leads to changes in hormone levels. The hypothalamic-pituitary-testicular axis controls the release of sex hormones, and aging alters steroid hormone levels affecting spermatogenesis (23).

In this study, DFI correlated negatively with PR%. DNA damage includes base mismatches produced during replication, generation of base-free sites, base modifications (oxidation, alkylation, deamination), formation of adducts, intrachain crosslinks, pyrimidine dimers, DNA single-strand breaks (SSBs) and double-strand breaks (DSBs) (24). The relevant mechanisms are as follows: 1. sperm DNA fragmentation correlates positively with ROS overproduction (25). Elevated ROS levels damage sperm cell membrane structure and affect sperm motility (20). In animal models, high concentrations of ROS oxidatively damage mitochondrial function and accelerate mitochondria-dependent apoptosis, which may decrease sperm motility (26,27). 2. Sperms can undergo apoptosis after ejaculation, which can damage mitochondrial integrity and result in nuclear envelope defects and nuclear fragmentation, affecting fertilization potential (28). Endogenous apoptosis is caused by ROS-mediated activation of pro-apoptotic factors, which leads to leakage of cytochrome C from the mitochondrial membrane and DNA damage (29). Exogenous apoptosis is initiated by the activation of

Table 3. Differences in PR% among C677T genotypes in the same age group.

Age (years)	C677T genotypes	N (%)	PR ≥ 32%	10% ≤ PR <32%	1% ≤ PR <10%	PR < 1%	$\chi 2$	*P*
	CC	50 (30.9)	46	3	0	1	7.986	0.175
<30	CT	76 (46.9)	71	4	1	0		
	TT	36 (22.2)	30	6	0	0		
	CC	140 (30.4)	118	22	0	0	5.890	0.437
30~35	CT	205 (44.5)	181	20	3	1		
	TT	116 (25.2)	101	14	1	0		
	CC	63 (28.4)	51	10	2	0	3.187	0.882
>35	CT	95 (42.8)	82	9	3	1		
	TT	64 (28.8)	52	9	3	0		

PR%: progressive motility.

Table 4. Differences in DFI among C677T genotypes in the same age group.

Age (years)	C677T genotypes	N (%)	DFI	*F*	*P*
	CC	50 (30.9)	16.96±8.35	1.026	0.361
<30	CT	76 (46.9)	15.32±7.83		
	TT	36 (22.2)	17.36±8.48		
	CC	140 (30.4)	17.22±8.95	0.971	0.380
30~35	CT	205 (44.5)	18.61±9.91		
	TT	116 (25.2)	17.78±8.54		
	CC	63 (28.4)	20.75±10.62	0.444	0.642
>35	CT	95 (42.8)	19.74±9.80		
	TT	64 (28.8)	21.27±11.07		

DFI, DNA fragmentation index.

Fas protein receptors on sperms. Leukocytes expressing the FasL ligand bind to Fas receptors, leading to the activation of pro-apoptotic proteins, which in turn disrupts mitochondrial pathways and causes DNA damage (29,30). High expression of Fas/Fasl induces testicular cell apoptosis in mice (31). In summary, the higher the level of sperm DFI, the higher the ROS levels and activation of apoptotic pathways, which may affect sperm motility, resulting in low PR%.

Mechanism of action of MTHFR

The main role of MTHFR is to convert 5,10-methylenetetrahydrofolate in the folate metabolic pathway to 5-methyltetrahydrofolate, which performs several biological functions (10). First, folic acid is metabolized to S-adenosylmethionine (SAM) via a single carbon cycle. SAM is the main methyl donor for DNA methyl transferases (32). Second, 2.5-methyltetrahydrofolate enters the methyl transfer pathway and indirectly provides methyl groups for DNA and protein methylation via Hcy remethylation; it maintains Hcy at a low level in the blood to avoid hyperhomocysteinemia (HHcy) (33). These processes can cause DNA methylation, resulting in abnormal chromosome segregation, which affects the production and function of sperms (34). Third, as an antioxidant, folic acid can resist oxidative stress. However, when its plasma levels are not sufficiently high to effectively block oxidative stress, the Hcy produced mediates DNA damage, leading to sperm DNA damage (34). Overall, the folate metabolic pathway affects DNA methylation and oxidative stress, resulting in impaired sperm function.

MTHFR C677T genotype and its relationship with PR% and DFI

Survey of the MTHFR C677T genotype

In various ethnicities: In a study that included ethnicities such as Whites, Hispanics, and Blacks of USA, Asians, and Amerindians, the frequency of C677T was highest among the Italians and Hispanics of the USA, lowest in the Blacks of some areas of the USA, and intermediate in Sub-Saharan Africans, and Whites of Canada, Britain, and the USA (35). In Italy, the 677TT genotype accounted for 27.6% (36). Among Hispanic, 677TT accounted for 17.4% (37). The 677TT genotype frequency is 11.6% in non-Hispanic Whites (38). For Blacks and Indians in Brazil, the frequency of occurrence of 677TT is only 1−2% (38,39). In summary, the frequency of the *MTHFR* C677T genotype differs with countries, regions, and races.

In infertile individuals: The results of *MTHFR* C677T genotype detection in this study were as follows: wild type (CC type), 253 cases (29.9%); heterozygous genotype (CT type), 376 cases (44.5%); mutant homozygous genotype (TT type), 216 cases (25.6%). The participants of this study were selected in infertility clinics; hence, the *MTHFR* C677T genotype ratio may be close to that of infertile men in Asia. In Korea, the frequencies of detecting the 677TT genotype in fertile and infertile men were 12.88% and 16.89%, respectively. The C677T heterozygous (CT) and homozygous (TT) genotypes were only associated with azoospermia and severe oligoasthenospermia in cases of unexplained infertility (40). In Iran, the CC, CT, and TT genotype frequencies of C677T in cases of idiopathic oligoasthenoteratozoospermia (OAT) were 35.4%, 48.8%, and 15.8%, respectively, whereas, in the control group, these frequencies were 43.9%, 45.1%, and 11%, respectively. Sperm motility in the TT group was significantly lower than that in the CC group. However, total serum Hcy and folate concentrations of the OAT and control groups were similar, and the difference was not statistically significant (41). This suggests that existing mechanisms cannot completely explain the mechanism via which *MTHFR* C677T polymorphism increases the risk of infertility, which warrants further investigations.

In this study, we grouped the participants according to age and genotype, and then compared the differences in DFI and PR%. We did not observe any significant difference in DFI and PR% with *MTHFR* C677T polymorphism. Results obtained from studies conducted in Poland (42) and France (43) showed that the frequency of the *MTHFR* C677T polymorphism did not differ significantly between the case (male infertility) and control groups and that there was no correlation between male infertility and the presence of this polymorphism. Cornet et al. (44) suggested that SDF does not increase in homozygous or heterozygous carriers of *MTHFR* C677T, where a critical threshold of 20% was used for sperm nucleus decondensation index and SDF. Our conclusions are consistent with the results of Cornet et al.

Limitations of the study

As the subjects of this study were men from the hospital's infertility clinic, we concluded that the *MTHFR* C677T polymorphism was not associated with PR% and DFI in infertile men. The distribution of the *MTHFR* C677T genotype differs between infertile and fertile populations (40,41). Therefore, the effect of *MTHFR* C677T polymorphism on sperm of healthy men has to be investigated further.

Studies have reported that if a large amount of folic acid is obtained from diet or drugs, the sperm quality of even TT carriers will not deteriorate to cause infertility (45). Therefore, folic acid intake should be included or folic acid level should be directly measured in future studies to improve the reliability of the correlation analysis.

Prolonged abstinence from sex negatively affects sperm motility and sperm DNA integrity. The longer the abstinence, the worse the sperm motility and the higher the sperm DFI level, i.e., the sperm integrity is poor (46). In this study, abstinence days were limited to 2−7 days; however, the data was not classified further, which may affect the accuracy of the results.

Sperm concentration should have been included in the study. However, the SCD method used for analyzing sperm DNA integrity in this study requires $\geq 5 \times 10^6$ sperms, because of which the data on sperm concentration $<5 \times 10^6$ will be lost, which will affect the accuracy of the results. If flow cytometry is used to measure sperm DNA integrity, sperm concentration can be included in the study, which will make the study more comprehensive.

In this study, we observed that age exerted a negative effect on PR% and sperm DNA integrity. Age correlated positively with DFI and negatively with PR%. For infertile men, *MTHFR* C677T polymorphism was not associated with sperm PR% and sperm DNA integrity. The above observations indicate that the relationship between *MTHFR* C677T polymorphism and infertility may not only depend on ethnic differences but also on factors such as

folic acid intake and data processing methods. We believe that the observations of our study can be used to avoid the influence of adverse factors on fertility in the clinic. Based on the relationship between *MTHFR* polymorphism and infertility, male folic acid intake can be determined to reduce the incidence of male infertility. We expect that in the future, more comprehensive, multi-ethnic, and multi-regional research will reveal the relationship between *MTHFR* C677T polymorphism and male infertility.

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