Association of methyltetrahydrofolate reductase gene mutation, homocysteine level with semen quality of Iraqi infertile males

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Abstract

Background: Infertility is a very common condition and almost 50% of cases are due to male factors. Several genetic and environmental factors are responsible for the poor quality and reduced number of sperms in several cases of infertility. The present study was designed to investigate the association between semen parameters, homocysteine, and the risk of C677T polymorphism of MTHFR gene in infertile males of Iraqi population.

Methods: This Case–control study has been conducted from February 2019 to July 2021 at a molecular laboratory in the Anatomy and Histology Department/college of Medicine/University of Kufa/Najaf/Iraq. It was composed of 353 infertile male patients. They were divided into five groups: 90 azoospermic, 84 oligospermia, 64 asthenospermic, 50 oligoasthenospermic, and 65 teratospermic with an age range 20–46 years compared with 100 fertile males as control with age range 21–49 years. In order to detect homocysteine levels, we used Hcy ELISA Kit. C677T mutation of MTHFR gene was employed by PCR–RFLP technique.

Results: Our data revealed three genotypes of MTHFR C677T, 167 (47.3%) subjects had CC genotype, 116 (32.9%) subjects had CT genotype and 70 (21.1%) subjects had TT genotype. Furthermore, T allele was associated with higher risk of infertility in all patients groups for any genetic model. In total infertile subjects (codominant model: CT vs. CC, \( OR = 2.0, 95\% \text{ C.I} = 1.2–3.3, \text{P} = 0.011; \) TT vs. CC, \( OR = 4.8, 95\% \text{ C.I} = 3.3–8.2, \text{P} = 0.0003; \) dominant model: CT + TT vs. CC, \( OR = 2.8, 95\% \text{ C.I} = 1.7–4.5, \text{P} = 0.0001 \)). Oligoasthenospermic patients associated with higher risk in CT heterozygous genotype (OR = 2.8, 95% C.I = 1.0–4.9, P = 0.03) and TT homozygous of mutant allele (OR = 6.3, 95% C.I = 1.9–9.2, P = 0.002). Homocystein level was elevated in all infertile groups when compared with control group (P < 0.01), but the elevation was marked in oligoasthenospermia group. As well as, the level of Serum Hcy exhibited the highest value in TT mutant genotype (39.7 µmol/ml) followed by CT genotype (28.5 µmol/ml) while the lowest level of Hcy recorded in CC genotype (14.6 µmol/ml) for oligoasthenospermia group.

Conclusions: By relating the MTHFR C677T gene mutation with a higher homocystein level, the results showed that Iraqi males with this mutation are more likely to suffer from infertility.

Keywords: MTHFR C677T gene, Mutation, Homocystein, Infertility, PCR–RFLP, Iraq

Background

Infertility is a common health problem, affecting approximately 15–20% of couples who approaching childbearing age. About 50% of infertility due to male factors [1]. About 12% of men suffer from male infertility and approximately 15–30% of male infertility is attributed to genetic abnormalities, which include Y chromosome
microdeletions, chromosomal aberrations, translocation, and single gene mutation. Several factors may contribute to sperm quality and quantity reductions in some infertile men, including deleterious gene mutations in key genes which are involved in testicular function [2, 3].

Mutations in genes involved in folate metabolism may influence spermatogenesis. The methylenetetrahydrofolate reductase gene (MTHFR), which is involved in folate metabolism, has been associated with abnormal spermatogenesis [4]. MTHFR is associated with an important pathway of folate-mediated methyl group metabolism. It is also involved in metabolizing thymine and purine, which are vital for dividing male germ cells to synthesize DNA. The 5,10-Methylenetetrahydrofolate is irreversibly reduced to 5-methyltetrahydrofolate by MTHFR, leading to the synthesis of methyl donor, which is required for methionine synthesis from homocysteine. Similarly, methionine forms S-adenosylmethionine, which is involved in a number of cellular reactions, including DNA, RNA, and histone methylation [5, 6].

Homocysteine accumulation and inappropriate methylation lead to DNA damage in spermatozoa which may reduce semen quality, affecting concentration, motility, and morphology of sperm [7, 8]. Therefore, deficiency in folate intake or mutation(s) in the folate pathway enzymes may result in aberrant DNA synthesis and methylation [9].

In adults testis of a mice, the activity of MTHFR is higher than other organs, representing its role in spermatogenesis [10, 11]. Therefore, alterations in the MTHFR gene could change the process of spermatogenesis and predispose carriers to infertility [12, 13].

MTHFR is a gene that regulates the synthesis of methylenetetrahydrofolate enzyme, is a key enzyme in homocysteine and folate metabolism, which acts as a carbon donor for remethylation of homocysteine to methionine. This enzyme reduces 5, 10-methylenetetrahydrofolate to 5, 10-methylenetetrahydrofolate, which is the predominant form of folate in the bloodstream. MTHFR gene is located on chromosome 1 p36.3, composed of 11 exons [14]. A total of 65 polymorphisms have been found in the MTHFR gene. The C677T (rs1801133) polymorphism results in a reduction of enzyme activity by 35%, which adversely affects nucleic acid metabolism [15].

Numerous studies have investigated the relationship between MTHFR gene mutation and male infertility, however, the conclusions are controversial [1, 4–6, 9, 16]. The cause of these conclusions can be attributed to small sample size, definition of infertility, and confounding factors such as ethnicity and race [17]. In the current study, we aim to identify a possible relationship between idiopathic sperm disorders, homocysteine concentration, and the MTHFR C677T mutation in the Iraqi population.

**Methods**

All participants provided informed consent before specimens were taken. The medical ethics committee at the University of Kufa/Faculty of Medicine approved the consent protocol for this study. During the period from February 2019 to July 2021, total of 353 infertile male patients and 100 age-matched fertile males were examined for this study. All patients were tested for seminal fluid analysis. Culture media preparation, Giemsa stain, and chromosome cytotype have been published elsewhere [18–20].

We selected the patients for the study based on andrology tests, including karyotyping and microdeletions of the Y chromosome. The study excluded individuals with cryptorchidism, atrophic azoospermia, numerical or structural chromosomal abnormalities, or Y-chromosome microdeletions.

A current case–control study was conducted in the Fertility center of Al-Sadder teaching Hospital and in the laboratories of the Faculty of Medicine/Kufa University in the Province of AL-Najaf /Iraq. The 353 infertile male patients were divided to five groups according to WHO 2020 criteria of semen parameters (colour, pH, semen volume, liquefaction time by macroscopic analysis while sperm count, sperm motility, and sperm morphology by using microscopic analysis). It is considered normal for men with ≥16 million sperms/milliliter [18]. There were 90 men with lack of sperm as azoospermic, 84 men with sperm count <16 million/milliliter were oligospermic, 64 of men with total motility <42% of sperm activity were asthenospermic, 65 of men with <54% abnormal sperm morphology were teratospermic and 50 of men were oligoasthenospermic, there age range (20–46 years) compared with age matched 100 normal fertile males as control with age range (21–47 years).

Semen examinations were conducted twice a month for each participant after three days of abstinence, after centrifugation for 10 min at 1006 ×g, semen samples were analyzed, based on reference values published by the World Health Organization, the mean values of seminal fluid analyses were recorded and used as average results [18]. From all participants a total of five milliliter venous blood samples were collected. one ml of blood sample was drowning in EDTA tube for DNA extraction, another amount of blood samples were centrifuged for 10 min at 1006 ×g, and serum were separated and stored at –17 °C until the assayed was performed. Total plasma homocysteine was analyzed according to the manufacturer procedure for determination by using microplate enzyme immunoassay ELISA kit method of Biorad laboratories. The amplification of DNA was done by polymerase chain reaction (PCR) using primers 5’TGA AGG
patients and control groups, the results were indi-
Hardy Weinberg equilibrium (HWE) among different
locus of the MTHFR gene were significantly
amine for the C677T locus, and 70 subjects (21.1%) homozygous for
CC genotype, 116 subjects (32.9%) were heterozygous
167 subjects (47.3%) were homozygous for normal allele
test of chi-square (X²) was done for analyzed the
groups for any genetic models; in total infertile
subjects (codominant model: CT vs. CC, OR = 2.0,
95% C.I = 1.2–3.3, P = 0.011; TT vs. CC, OR = 4.8, 95%
C.I = 3.3–8.2, P = 0.0003; dominant model: CT + TT vs.
CC, OR = 2.8, 95% C.I = 1.7–4.5, P = 0.0001). Also the
same table revealed that oligoasthenospermic patients
associated with higher risk in CT heterozygous genotype
(OR = 2.8, 95% C.I = 1.0–4.9, P = 0.03) and TT homozy-
gous of mutant allele (OR = 6.3, 95% C.I = 1.9–9.2,
P = 0.002).
As shown in Table 2, homocysteine level of each infer-
tile group according to MTHFR gene polymorphism
(rs:1801133) genotypes (co-dominant model), was com-
pared with control group and the results showed sig-
nificant increase in Hcy levels in infertile groups when
compared with control group (P < 0.01). There were high
levels of serum Hcy in homozygous genotype (TT) when
compared with heterozygous genotype (CT) and nor-
mal allele genotype (CC) in all patient groups. But the
highest levels of Serum Hcy was recorded in oligoasthe-
nospermia group (31.5 µmol/ml) followed by the azoo-
spermia group (28.7 µmol/ml), asthenospermia group
(25.9 µmol/ml), teratospermia group (24.2 µmol/ml), and
oligospermia group (21.3 µmol/ml), whereas the lowest
level of Hcy appeared in the control group (12.1 µmol/
ml).
As well as, the level of Serum Hcy exhibited the highest
level in oligoasthenospermia group of TT mutant geno-
type (39.7 µmol/ml) followed by CT genotype (28.5 µmol/
ml) while the lowest level of Hcy was recorded in the CC
genotype (14.6 µmol/ml).

Results
A total of 353 infertile men patients and 100 normal fer-
tile men were enrolled in the current study. The patient's
group was divided into five subgroups: 90 azoospermia,
84 oligospermia, 64 asthenospermia, 50 oligoasthe-
nospermia, and 65 teratospermia.
The test of chi-square (X²) was done for analyzed the
Hardy Weinberg equilibrium (HWE) among different
patients groups and control groups, the results were indi-
cated that an observed and expected frequencies were
consistent among the three genotype groups (P > 0.05)
indicating that the frequency of this sample was repre-
sentative the Iraqi population.
Three genotypes of CC, CT, and TT were found in the
locus 677 of the MTHFR gene in the Iraqi population,
the allele distribution along with genotype frequencies
of three genotypes are shown in Table 1. Accordingly,
167 subjects (47.3%) were homozygous of normal allele
CC genotype, 116 subjects (32.9%) were heterozygous
of CT genotype, and 70 subjects (21.1%) homozygous of
mutant allele TT genotype. The genotype frequencies of
the C677T locus of the MTHFR gene were significantly
different when infertile men compared with the control
group. T allele associated with greater risk of infertility in
all patients groups for any genetic models; in total infertile
subjects (codominant model: CT vs. CC, OR = 2.0,
95% C.I = 1.2–3.3, P = 0.011; TT vs. CC, OR = 4.8, 95%
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CC, OR = 2.8, 95% C.I = 1.7–4.5, P = 0.0001). Also the
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Discussion
Spermatogenesis is a complex development process that
involves mitosis, meiosis, and spermiogenesis. Methyl
group formation mediated by folate, is one of many
pathways regulating this complex process. Besides pro-
viding the bases for DNA synthesis in rapidly dividing
male germ cells, this pathway is also critical for the
removal of homocysteine and several methylation reac-
tions such as methylation of DNA, RNA, histones, and
other molecules [5, 21]. Folate deficiency is associated
with various illnesses including male infertility [22]. As
a result of changes in folate status, spermatogenesis can
be affected by DNA hypomethylation and uracil misin-
corporation during DNA synthesis, resulting in errors
in DNA repair and chromosomal anomalies [23]. There
is clear evidence that hyperhomocysteinemia increases
the risk of cardiovascular disease [24] and neural tube
defects [25, 26], but its effects on spermatogenesis
remain unclear. The epigenetic architecture of sperm
DNA has been studied and it has been shown that sperm DNA, histones methylation patterns are vital for normal sperm function, as well as there is significant association between methylation of certain genes and sperm concentration, motility, and morphology [27–33]. Numerous studies have suggested that mutations in the MTHFR C677T gene may be associated with decreased sperm counts in the human that lead to male infertility in a number of populations [34–37].

Infertility in men has been studied in different populations to estimate the association between homocysteine levels, MTHFR gene and infertility. In fact, the results were conflicted due to differences in sample size,

### Table 1 Genotype frequencies of MTHFR (rs1801133) gene mutation in the studied subjects

| Patients group | Genotype | Patients n (%) | Control n (%) | OR* (95%CI) | P value |
|----------------|----------|----------------|---------------|-------------|---------|
| Azoospermic n = 90 | CC | 41(45.5) 70(70) | 1 | | |
| | CT | 30(33.3) 25(25) | 2.0(1.1–3.9) | 0.03 |
| | TT | 19(21.1) 5(5) | 4.5(2.3–10.7) | 0.0005 |
| | CT+TT | 49(54.4) 30(30) | 2.8(1.5–5) | 0.0007 |
| Oligospermic n = 84 | CC | 42(50) 70(70) | 1 | | |
| | CT | 25(29.7) 25(25) | 1.7(0.8–3.3) | 0.1 |
| | TT | 17(20.2) 5(5) | 3.7(1.9–6.6) | 0.002 |
| | CT+TT | 42(50) 30(30) | 2.3(1.2–4.2) | 0.006 |
| Asthenospermic n = 64 | CC | 31(48.9) 70(70) | 1 | | |
| | CT | 24(37.5) 25(25) | 2.2(1.0–4.4) | 0.03 |
| | TT | 9(14) 5(5) | 4.0(1.2–7.1) | 0.01 |
| | CT+TT | 33(51.5) 30(30) | 2.5(1.3–4.8) | 0.006 |
| OligoAsthenospermic n = 50 | CC | 22(44) 70(70) | 1 | | |
| | CT | 18(36) 25(25) | 2.8(1.0–4.9) | 0.03 |
| | TT | 10(20) 5(5) | 6.3(1.9–9.2) | 0.002 |
| | CT+TT | 28(56) 30(30) | 2.9(1.4–6.0) | 0.002 |
| Teratospermic n = 65 | CC | 31(47.7) 70(70) | 1 | | |
| | CT | 19(29) 25(25) | 1.7(0.8–3.5) | 0.1 |
| | TT | 15(23.1) 5(5) | 5.0(2.2–9.8) | 0.0006 |
| | CT+TT | 49(75) 30(30) | 3.0(1.6–4.8) | 0.0001 |
| Total infertile subjects | CC | 167(47.3) 70(70) | 1 | | |
| | CT | 116(32.9) 25(25) | 2.0(1.2–3.3) | 0.011 |
| | TT | 70(21.1) 5(5) | 4.8(3.8–8.2) | 0.0003 |
| | CT+TT | 201(56.9) 30(30) | 2.8(1.7–4.5) | 0.0001 |

*OR* adjusted odds ratio, 95% CI 95% confidence interval, n number

### Table 2 Comparison of serum homocystein concentrations (µM) with patients groups

| Patients group | Genotype | Hcy concentration Mean ± SD |
|----------------|----------|-----------------------------|
|                | CC       | CT             | TT             |
| Azoospermic    | 12.4 ± 2.7 | 22.5 ± 3.2  | 38.7 ± 5.1   | 28.7 ± 6.1* |
| Oligospermic   | 10.2 ± 4.1 | 19.6 ± 3.9  | 35.5 ± 3.6   | 21.3 ± 4.3* |
| Asthenospermic | 11.3 ± 6.1 | 23.3 ± 7.1  | 37.6 ± 8.2   | 25.9 ± 9.4* |
| OligoAsthenospermic | 14.6 ± 3.5 | 28.5 ± 7.4  | 39.7 ± 6.5   | 31.5 ± 7.6* |
| Teratospermic  | 12.7 ± 3.8 | 22.9 ± 6.3  | 29.6 ± 4.6   | 24.2 ± 4.3* |
| Control group (n = 100) | 9.2 ± 2.3 | 10.2 ± 2.6 | 13.7 ± 3.2 | 12.1 ± 2.6* |

*P < 0.01
ethnicity, race, geographic variations, genetic variation, and exposure to environmental risk factors [38–40].

Up to our knowledge, there is no data regarding the relationship between the MTHFR C677T polymorphism, homocysteine and semen parameters in Iraqi infertile patients, so this study is designed to assess the relationship of MTHFR gene polymorphism, homocysteine levels, and semen parameters quality in Iraqi population.

The results revealed that MTHFR C677T gene mutation is a risk factor for male infertility in our population, also, it appeared high homocysteine levels in azoospermic, oligospermic, asthenospermic, teratospermic, and oligoaethnospermic patients groups. Highest level was in oligoaoethnospermic group. TT mutant genotype was more than CT genotype while the lowest level of homocysteine recorded in CC genotype.

Our results were consistent with the Egyptian study which one [41] out of four studies in Africa showed a high association of 677 T with men's infertility [41–44]. A two Iranian studies were found that carriers of the 677 T allele (CT and TT genotypes) at a higher risk of infertility than individuals with other genotypes [45, 46], In Chinese men with azoospermia and severe oligoazoospermia, an SNP in the MTHFR gene appeared to be associated with infertility [47], an Italian study of Lombardo et al. [48] who recorded that a function of C677T mutation in MTHFR leads to hyperhomocysteinemia, is associated with erectile dysfunction mainly in homozygous 677 TT patients. Also in France Montjean et al. [49] and Ménézo et al. [50] was recorded the MTHFR 677TT genotype is combined with significantly increased plasma homocysteine levels, in Netherlands Boxmeer et al. [51] was recorded increased sperm damage by increased homocysteine and decreased folate in seminal plasma. A number of Asian studies [52–55] expressed this association, Mutant genotypes are significantly associated with fertility by Gupta et al. [56] and Kumari et al. [57] studies in India, another study in Italy that conducted by Paracchini et al. [58] was found the risk of infertility increased with TT genotype in the C677T locus of MTHFR gene.

In contrast, the MTHFR 677 T mutation was found to be protective for Moroccan infertile men [59]. Other studies in the Indian population [60, 61] were deserved no significant differences of C677T variants between fertile and infertile males. Celia et al. [62] found no evidence for an association between C677T polymorphism in enzymes involved in folate metabolism and reduced sperm counts in the French population.

Conclusion
By relating the MTHFR C677T gene mutation with a higher homocysteine level, the results showed that Iraqi males with this mutation are more likely to suffer from infertility. Additional data are needed to confirm our conclusion and provide an important relationship between genes and the environment that affects male infertility risk.

Abbreviations
MTHFR: Methyltetrahydrofolate reductase; Hcy: Homocysteine; ELISA: Enzyme-linked immunosorbent assay; PCR–RFLP: Polymerase chain reaction-restriction fragment length polymorphism; C: Cytosine; T: Thymine; OR: Odds ratio; CI: Confidence interval; P: Probability; X²: Chi-square; HWE: Hardy Weinberg equilibrium; SPSS: Statistical package for the social sciences.

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Authors' contributions
A substantial and intellectual contribution has been made by each author and the work has been approved for publication by all of them. AM and SM contributed to the conceptualization. AM, SM and SA were involved in formal analysis and literature search. Sample collection, data acquisition and methodology; biochemical and genetic analyses were done by AM and SM. Statistical analysis was calculated by AM. AM, SM and SA contributed to the manuscript editing and review. AM and SM contributed to the manuscript revision. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on request.

Declarations
Ethics approval and consent to participate
The study was conducted after gaining approval from the medical ethics committee (Kufa Faculty of Medicine/University of Kufa, Najaf) vide Reference # MEC-09 dated 19.2.2019. Written informed consent was taken from the study participants.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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