Original Article

Investigation of the Relationship Between Polymorphism of *MTHFR* Gene & Male Infertility in Some People at Thi – Qar Province

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Abstract

Background: The gene known as methylenetetrahydrofolate reductase (*MTHFR*) is control of the production and methylation of deoxynucleic acids. Male infertility has been linked to the *MTHFR* polymorphism (C677T). **Objective**: a current study on male infertility was carried out to ascertain whether the *MTHFR* polymorphism (C677T) is connected to male infertility. The study included 30 male infertility patients aged between (26-58) years and 20 individual healthy control groups. The studied ages were distributed into three groups. The major group was (26 – 36) years, and the percentage was (40%). The second group was (37 – 47) years, and the rate was (26.67 %); the third group was (48 – 58) years, with a percentage (of 33.33%). The last group is the most prevalent among the three groups. **Method**: DNA was isolated, and Restriction Fragment Length Polymorphism – Polymerase Chain Reaction (RFLP-PCR) was done by using specific primers to the *MTHFR* gene. **Results**: the results indicated to the presence of mutations in 15 out of 30 patients after using restriction enzymes *Hinf*. Statistical analysis showed a correlation between male infertility and the emergence of *MTHFR* C677T gene mutation paralleled through the control group. **Conclusion**: When we compared the patient's group and the healthy group, we established there is a correlation between *MTHFR* C677T gene polymorphism and the incidence of male infertility in our study population.

Keywords: *MTHFR*, polymorphism, Infertility, RFLP, PCR.

Introduction

Infertility is the lack of ability to imagine following a year of consistent, unprotected communication, is a significant health problem. Approximately 10-15% of worldwide affect by infertility, and the reason for half of these occurrences is male infertility.[1] Male infertility is a important case faced by wed couples worldwide; the ratio of infertile couples is gradually growing.[2] Male infertility is a diverse disease caused by several environmental and genetic reasons leading to spermatogenesis flaws.[3] Genetic causes, such as single gene mutations and chromosomal aberrations, play essential roles in males infertility in addition to other risk factors as environmental factors and lifestyle. *MTHFR* is the essential enzyme of folate metabolism. Also, the *MTHFR* protein causes the synthesis of RNA and DNA and the re-methylation of homocysteine).[4] The enzyme activity of *MTHFR* influences by gene polymorphism.[5] The polymorphism of *MTHFR* perhaps a possible factor for male infertility.[6] *MTHFR* gene, positioned on 1p36.3, the result of transcription is a 77 KDa,[7] *MTHFR* has two common polymorphisms. The first polymorphism at exon (4) is the *MTHFR* C677T mutation, where the alanine change to amino acid valine at location 226 in the protein. While second mutation positioned at exon(7) is (*MTHFR* A1298C), where the amino acid glutamate change to the amino acid alanine.[8] Depending on their locations, single nucleotide polymorphisms can influence

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the gene's structure and expression. Gene expression is impacted by polymorphism in the promoter region but not by exonic polymorphism alters a protein's function and structure.[9] The *MTHFR* Polymorphism shows an central role not only in male infertility but as well as in other diseases. *MTHFR* gene polymorphisms may show central role in enlargement of diabetes-associated disorders and cardiovascular diseases, such as nephropathy and retinopathy.[10] Also, *MTHFR* C677T polymorphism plays an important role in women's pregnancy.[11] One study was aimed to test the relationship between the polymorphism of *MTHFR* gene (C677T) and colorectal cancer vulnerability, where the results presented the SNP is a positive factor to the patients.[4] Numerous studies have scanned the association between the risk of male infertility and *MTHFR* polymorphisms. Though, the effects stay contentious and earlier studies have generally been lesser. Many studies support that polymorphism in *MTHFR* is an essential genetic reason in male infertility, such as Alhumaydhi *et al*.[12], where the authors concluded that polymorphism in *MTHFR* (677C>T) gene is an significant reason for male infertility. In the present study, we want to explain if there is evidence of a correlation between *MTHFR* gene polymorphism and infertility in male in some males in Thi – Qar Province by using the RFLP-PCR technique after extracting DNA and then using the restriction enzyme *HinfI***.**

Materials and Methods

The present study was done during the period from September 2020 - September 2021 in Thi-Qar Province to investigate the effect of Methylene tetrahydrofolate reductase gene polymorphism on some male infertility. The equipment were used in this study showed in Table (1).

Table 1: The Apparatus which Were Used in the Study.

Equipment	Company	Origin
Autoclave	Hiryama	Japan
Centrifuge	Sigma	Germany
EDTA tubes	AFCO – Dispo	Jordan
Electrophoresis	Atto	Japan
Eppendroff tubes	AFCO – Dispo	Jordan
Graduated class cylinder	Marienfeld	Germany
Incubator	Fisher Scientific	USA.
Microcentrifuge	Kubota 3220	Japan
Micropipettes	Volac	England
Refrigerator	National	Japan
Sensitive Balance	Persica XB 220A	Switzerland
Syringe	AFCO – Dispo	Jordan
Microwave	Biocote	U.K
Thermo Cycler	Esco	U.S.A
UV light transilluminator	Viber Lourmat	U.S.A
Vortex	Grant- Bio	U. K
Water Distillatory	Daihan Lab	Korea

The chemicals used in this study are showed in Table (2)

Table 2: The Materials which Were Used in the Present Study.

Materials	Corporation	Origin Country
Ethanol	GCC	U . K
Agarose	Promega	U . S . A
Bromophenol Blue	Thomas Baker	India
Ethidium Bromide	Promega	U . S . A
TBE buffer	Thermo Fisher Scientific	U . K

The Biological materials and kits which were used in the study are showed in Table (3)

Table 3: The kits and Biological Materials which Were Used in the Present Study.

Materials	Corporation
R&F primers	Biocorp
Restriction enzymes *Hinf*	Sigma
DNA Ladder Marker	Bioneer
DNA extraction kit	Gene aid
Proteinase K	Promega
Master Mix	Bioneer

The blood samples were obtained by venipuncture from studied groups. Three ml of blood were drawn by syringe. Samples were put in an EDTA tube for DNA extraction by using kit extraction (Gene aid company). Then detecting DNA by the electrical relay(Electrophoresis) by using the gel (Agarose).This includes the following steps: 1- TBE buffer– 1X (25) ml was added to a sterilized beaker. Agarose 0.2 gm was added to the beaker. 2-
3- Agarose was boiling until it melted.
4- Adding ethidium bromide (0.5) to the melted gel and left cool at 50-60C.

After that, Preparing the Casting Agarose Gel

a. One of the cast endings contained the comb.
b. After sealing the cast's ends to prevent leaking, the agarose solution was poured inside and allowed to cool at room temperature.
c. After removing the comb, the cast was placed in an electrophoresis chamber.
d. The electric electrophoresis solution (1 X TBE buffer) was poured into the cavity device deportation as it rose 3-5 mm above the gel's surface.

DNA Loading and Electrophoresis Through the Following Steps

a. Addition 9 µl of DNA to 3 µl of the bromophenol blue stain, then it loaded in gel wells.
b. Electrodes of the apparatus were connected to the power source.
c. Electrophoresis apparatus was at80V and 120 mA then dye was migrates from the gel wells to cross.
d. The gel was located on a ultra-violate light and a photo was taken to it.

DNA Template of MTHFR Gene Prepared as Follows

- Primers (reverse& forward) as in Table (4).
- Master Mix.
- Sterilized D.W.

Table 4: Primers Sequences Used for the *MTHFR* Gene.

Primer	Primer sequences'
Forward	5 –TGA- AGG- AGA –AGG- TGT- CTG –CGG- GA-3
Reverse	5-AGG -ACG –GTG- CGG- TGA- GAG -TG-3

Forward and Reverse, distill water and DNA mixed in the master mix tube (5µl), as in Table (5).

Table 5: PCR Reaction for Amplification of *MTHFR* Gene.

Materials	Volume
Master Mix	five µl
Primers F&R	one µl for one gene
DNA	eight µl
Nuclease free water	five µl
20 µl	Total

After that, the tubes were placed in the thermal cycle, see Table (6).

Table 6: PCR Condition for Amplification.

Steps	Steps	Temp	Time	Cycle No
1	Denaturation 1	**94**	**1 min**	**35**
2	Annealing	**68**	**1 min**	**1**
3	Extension	**72**	**1 min**	**1**

The PCR products were separated on 1.5% agarose and imagined by UV under 302nm afterward staining with ethidium bromide. *Hinf* restriction site confirmed by incubating 3 Unit of *Hinf* and buffer solution (3µl) with (10µl) of the PCR products at 60 C overnight. Then the product was electrophoresis on agarose gel.

Results and Discussion

After extracting total DNA, products were electrophoresis on agarose gel on a 0.8% by using Bromophenol blue stain and visualized under UV light see Figure 1.

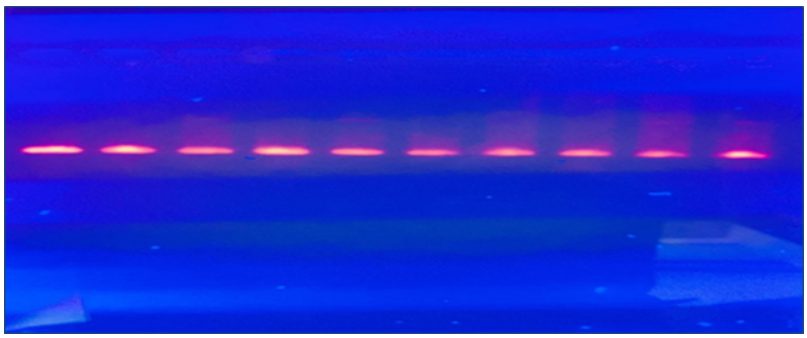

Figure 1: Gel Electrophoresis for DNA on 0.8 % Agarose.

Agarose gel electrophoresis for amplified *MTHFR* gene. on 2 % agarose gel and visualized at U.V. after stain by ethidium bromide, as in Figure 2.

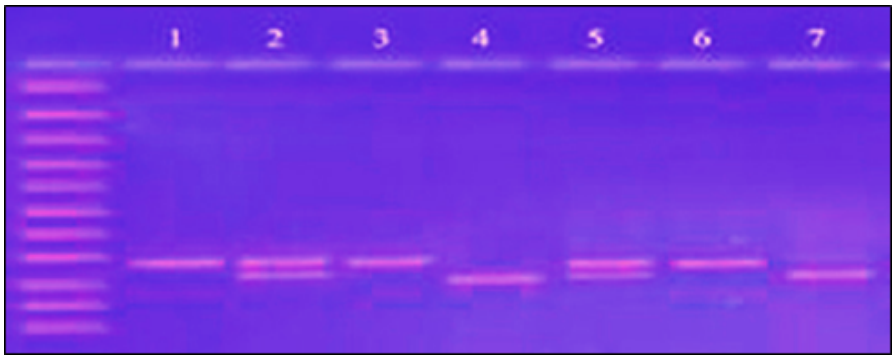

Figure 2: Amplified *MTHFR* Gene Gel Electrophoresis. On 2 % Agarose Gel for 1 Hour, 80V/cm, 1x Tris-Acetic Buffer). (DNA Ladder:100 - 2000bp). Product Band Lan 1, 3, and 6: (C/C) Wild Genotype of *MTHFR* Gene, 198 bp. Lan 2 and 5 (C/T) Heterozygous Mutant Genotypes 198, 175, and 23 bp). Lanes 4 and 7(T/T) Homozygous Mutant Genotypes, Bands 175 and 23 bp. Band 23 Below 50 bp.

This study presented a significant difference between age groups in cases and the *MTHFR* genotype, especially in the second age group (37-47) years when OR=1.5000 and CI = 0.2029 to 11.0882 see Table (7)

Table 7: The Relationship between *MTHFR* Genotype and Age Group in Cases.

Age Group	EM	HEM	OR	CI 95%
26 – 36	**4 (13.3 %)**	**8 (26.6 %)**	**1.0**	**_____**
37 – 47	**2 (6.6 %)**	**6 (20 %)**	**1.5000**	**0.2029 to 11.0882**
48 – 58	**6 (20 %)**	**4 (13.3 %)**	**0.333**	**0.0583 to 1.9066**

The results of this study offered a association between the genotypes of the *MTHFR* gene and the occurrence of male infertility, by way of the results presented a significant difference between cases and the healthy group, as in Table 8.

Table 8: Distribution of *MTHFR* Gene Polymorphism between Control and Patients Groups.

Genotype	Controls (%)	Patients (%)	OR	CI 95%
EM	12 (60%)	6 (20%)	1.0	---
HEM	7 (35%)	9 (30%)	2.5714	0.6396 to 10.3386
PM	1 (5 %)	15 (50%)	30.0000	3.1651 to 284.3543

EM: Homozygous (Extensive metabolizers).
HEM: Heterozygous (Hetero extensive metabolizer).
PM: Poor metabolizer.

So this study showed no correlation between genotypes in male smoking and the appearance of male infertility, as seen in Table (9)

Table 9: OR of Development for *MTHFR* Genotype Stratified by Status of Smoking.

Smoking Status	EM	HEM	OR	CI 95%
No-smokers	6 (60%)	4 (40 %)	1.0	-------
Smokers	12 (60 %)	8 (40 %)	1.0000	0.2124 to 4.7092

When we compared the age groups of patients, we noticed a significant difference in the second group when it went from (37-47) years, while there was no significant difference in the third group when it ranged from (48-58) years. This finding is perhaps because the male in the third group generally starts to reduce their fertility around this age when sperm quality decreases. Also, stay sitting for long times, tight clothes or working on a laptop for long periods can increase the temperature of the scrotum and may slightly reduce sperm production. The results of statistical analyses showed that there is a significant difference between mutant homozygous and

the mutant heterozygous. These results presented that there is a association between polymorphism of *MTHFR* gene and the occurrence of male infertility.

Our results support the participation of the *MTHFR* gene in male infertility in some populations in the Thi-Qar province. An explanation of this finding is auto-oxidation of *MTHFR* C667T polymorphism, causing the hydrogen peroxidase production by toxic reactive oxygen metabolites,[13] which could affect homocysteine-mediated DNA harm,[14] and might be befriend by an anti-oxidant delegate as folate. Folate accessibility is spurious connected with when polymorphic in *MTHFR*. Our results agree with Ren *et al*.[15] and Wang *et al*.[16] in the Chinese population. Also, our results agree with Shi *et al*.[17] but disagree with Raigani *et al*.[18], who didn't denote the *MTHFR* C677T gene polymorphism as a risk factor to male infertility in Iranian population. It is also among the studies that contradicted the results of our study[19] this study, which concentrated on the South Indian population, found no link between male infertility and the *MTHFR* C677-T gene variant. The *MTHFR* C677-T gene polymorphism also was not linked to infertility in South Indian men, according to this study.[20]

When we examine the distribution of genotypes of patients according to smoking, results show statistically non-significant differences between smokers and non-smokers for individuals with genotype homozygous. Also, genotype heterozygous showed no significant difference. These results suggest some risk factors, such as prolonged exposure to certain chemicals, industrial chemicals, herbicides, pesticides, paints, and organic solvents or genetic factors, may contribute to developing a problem in sperms count. Contact with heavy metals, lead or other metals may also cause male infertility. Also, x-rays or radiation exposure can decrease sperm production.

Conclusion

Folate is critical in DNA synthesis, methylation of the genome, and synthesis of protein. Folate shortage could miss the role of the mentioned actions and cause the proliferation of homocysteine, subsequent to great oxidative stress. This series of events is complicated in certain conditions, such as male fertility. Some enzymes and Methylenetetrahydrofolate reductase have a central role in folate metabolism and homocysteine pathways. We studied the relationship between the polymorphism of the *MTHFR* gene and male infertility. When we compared the patient's group and the healthy group, we established there is a correlation between *MTHFR* polymorphism and the incidence of male infertility. After using RFLP –the PCR technique to detect the mutation in the *MTHFR* gene. This consequence perhaps related to pathologies extra than male fertility, as cancer and cardiovascular diseases. The present study is restricted by the few sample; additional investigations on a bigger sample must be led to estimate the connotation of the *MTHFR* (C677T) SNP with male infertility. It is necessary to conduct studies to test the association of the genetic polymorphism of this gene on a sample of women because of the effect of it on male infertility, according to previous studies. Therefore necessary estimate this relationship in other populations to identify novel candidate genes to get a better understanding of the complex gene-to-gene interactions which have a deep effect on male infertility.

Conflict of Interest

The authors declare that they have no conflict of interest

Supplementary Material

All data generated or analyzed during this study are included in this published article.

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Risk Bias

The results showed that there was no risk of bias in this study.

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