Mutations in the Methylenetetrahydrofolate Reductase Gene and Methionine Metabolism in male infertility

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ABSTRACT

Background and Aim: *The human methylenetetrahydrofolate reductase (MTHFR) gene plays a crucial role in folate metabolism. Data regarding the influence of MTHFR gene polymorphisms on male fertility status are conflicting. The present study aimed to investigate the possible role of genetic variants of the MTHFR 677 C→T and 1298 A→C and the seminal plasma levels of S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH) in male infertility.* ***Patients & Methods:*** *The present study included 229 men attending the Andrology Outpatient Clinic, Mansoura University Hospital. The semen samples obtained from men were grouped according to the profile of seminogram into normozoospermic (N), oligoathenoteratozoospermia (OAT) and azoospermia (AZ). Spermatozoa were separated and the purified spermatozoa were used for assessment of acrosine activity by gelatinolysis. High Performance Liquid Chromatography equipped with a reversed-phase column-C18, and UV detector at 254 nm was used to separate SAM and SAH. Genomic DNA was isolated from peripheral blood leukocytes by Genta genomic DNA purification kit. MTHFR 677 C→T and 1298 A→C polymorphisms were analyzed using PCR, restriction enzymes and agarose gel electrophoresis.* ***Results:*** *The results of the current study showed that SAM, SAM/SAH ratio and acrosine activity index to be significantly decreased in OAT and AZ compared with normozoospermia. MTHR 1298AA and 677CC genotypes frequency was significantly higher in OAT and AZ groups when compared to N group..Also, SAH were significantly increased in MTHR 1298AA and 677CC genotypes.* ***Conclusion:*** *The polymorphisms in the MTHR A1298C and C677T gene were associated with abnormal sperm function, morphology and motility. Carrier of 1298AA and 677CC genotypes had higher level of SAH. It could be concluded that methionine metabolism is abnormal in infertile men denoted by impaired SAM and SAH levels. Further studies may be of benefit for new strategies in therapy for male infertility.*

INTRODUCTION

Couple infertility is a global health problem and according to the World Health Organization approximately one couple in seven is affected by fertility or subfertility problems[(1)]. Male infertility in humans has been acknowledged as the cause

of couple's inability to have children in 20-50% of total cases [2].

The metabolism of folate is critical for the maintenance of genome integrity due to its role in DNA synthesis, repair and methylation [3]. Methylenetetrahydrofolate reductase (MTHFR) catalyzes the reduction of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate (MTHF), the predominant circulatory form of folate and carbon donor for the re-methylation of homocysteine to methionine. Thus, MTHFR is thought to participate in the provision of nucleotides essential for DNA synthesis and repair. Methionine, in its activated form, S-adenosyl methionine (SAM), is the methyl donor of many biologic transmethylation reactions[4]. A decreased pool of methionine may therefore also affect DNA methylation and this is supported by the observation that some *MTHFR* variants are associated with DNA hypomethylation [5].

The human MTHFR gene has been mapped to chromosomal region 1p 36.3 [6]. Within the MTHFR gene, there were total number of 65 polymorphisms have been described [7]. The two common polymorphisms of the MTHFR gene are 677 C→T and 1298 A→C which are known to reduce the enzyme activity leading to a decreased pool of methyl-THF and associated with hyperhomo-cysteinemia, particularly in folate deficiency. The 677 C→T transition in the exon 4 causes an amino acid substitution from alanine to valine at codon 222 within the catalytic region of the enzyme. The 1298 A→C mutation resulting in glutamic-alanine

substitution in position 429 is within the proposed regulatory region of the methylenetetrahydrofolate reductase enzyme and may influence overall enzyme function, particularly in combination with the 677 C→T polymorphism[8].

MTHFR gene mutation C677T could influence its biochemical activity and studies have shown that TT (mutant) has 30% activity when compared with CC. Similarly, A1298C polymorphism also reduces enzyme activity but to a lesser degree than C677T [9]. Its deficiency could alter the synthesis of 5-methyl-tetrahydrofolate (5-MTHF), interrupt Hcy remethylation to methionine and cause hyperhomocysteinemia. Folate deficiency-linked hyperhomocysteinemia is a risk factor for many diseases including infertility[10]

MTHFR variants are known to influence disease processes and several studies have been reported on reduced enzyme activity and the susceptibility to different disorders, including vascular diseases, neural tube defects and cancer. Homozygous for MTHFR 677 C→T and 1298 A→C polymorphisms have been shown to reduce the risk of acute lymphoblastic leukemia [11].

Recent studies have reported diverse associations between MTHFR C677T polymorphism and infertility[12,13]. Low folate coupled with MTHFR SNPs can alter RNA/DNA synthesis and has the potential to be linked with infertility[14]. Animal model studies suggest that MTHFR plays a critical role in spermatogenesis due to exceptionally higher activity in adult

testis than other organs [15]. The aim of the current study is to analyze the association of 677 C→T and 1298 A→C polymorphisms of the MTHFR gene and S-adenosyl homocysteine and S-adenosyl methionine nucleosides on sperm function and hence their potential involvement as risk factors in male infertility.

MATERIAL & METHODS

The present study included 229 men attending the Andrology Outpatient Clinic, Mansoura University Hospital. The semen samples obtained from men were left for 30 minutes for liquefaction to occur, computer assisted semen analysis (Autosperm, Fertipro, Belgium)[16] was performed. Sperm morphology was evaluated by phase contrast microscope and sperm Mac stain (Fertipro, Belgium). The semen samples were grouped according to the recommendation of World Health Organization[17] into normozoospermia (N) (n= 90), oligoathenoteratozoospermia (OAT)(n= 64) and azoospermia (AZ) (n=75) . Spermatozoa were separated from WBCs by Sil- select gradient (Ferti Pro N.V., Industriepark Noord, Beerneme, Belgium) and the purified spermatozoa were used for assessment of acrosin activity by gelatinolysis and membrane integrity with hypoosmotic swelling test. Seminal plasma was obtained by centrifugation of two ml of semen sample at 4000 rpm for 15 minutes at 4°C for assessment of S-adenosyl methionine, S-adenosyl homocysteine and alpha glucosidase activity. Exclusion criteria for infertile men were hypertension,

diabetes mellitus and renal diseases. Patients with a history of varicocele, congenital abnormalities, urogenital infections and undescended testicles were excluded from the test after examination by a specialist. Informed consent regarding genetic testing was obtained from all study subjects.

1- DNA extraction and PCR for polymorphism of the MTHFR gene:

Genomic DNA was extracted from peripheral blood using Gentra genomic DNA purification kit. Each PCR was performed with 300 ng of DNA, 200 mmol./L of each dNTP 500 nmol./L of each primer and 2.5 units of Taq DNA polymerase (Amplitaq gold, Perkin Elmer cetus, Norwalk, conn). The regions containing 2 RFLPs within the MTHFR gene were amplified with Taq DNA polymerase.

For evaluation of MTHFR C677T polymorphism, the polymorphic site was amplified with primers [18]:
Forward (5′-TGAAGGAGAAGGTGTCTGCGGGA-3′)
Reverse (5′-AGGACGCTGCGGTGAGAGTG-3′). For PCR, the following procedure was used: initial denaturation for 3 minutes at 95 C°, and then 5 cycles were performed as follows: 94 C° for 1 minute, 64 C° for 1 minute and 72 C° for 30 seconds. Then, 30 cycles were performed as follows; 94 C° for 45 seconds, 62 C° for 45 seconds and 72 C° for 25 seconds. The PCR amplification was completed by a final extension at 72 C° for 7 minutes. The amplification yields a product 198 bp. Upon cleavage with 5 units of Hinf I (Boehringer Mannheim) for 16 hours at 37 C° and then, electrophoresed on 3 % agarose and

stained with ethidium bromide, and visualized under UV light. Allele T produced 2 bands at 175 bp and 23 bp whereas C allele remains uncut. The C677T genotypes were classified as CC, CT, or TT. For normal individual (C677C homozygous), agarose gel electrophoresis allows visualization of a 198 bp fragment. For T677T (homozygous patient), agarose gel electrophoresis allows visualization of two bands of 175 and 23 bp. For C677T (heterozygous patient), agarose gel electrophoresis allows visualization of three bands of 198, 175 and 23bp [19].

For evaluation of MTHFR A1298C polymorphism, the polymorphic site was amplified with primers:

Forward (5′-CTTTGGGGAGGTGAAGGACTAC TAC-3′)

Reverse (5′-CACTTTGTGACCATTCCGGGTTG -3′)

For PCR, the following procedure was used: initial denaturation for 2 minutes at 95 C°, and then 5 cycles were performed as follows: 95 C° for 1 minute, 55 C° for 2 minutes and 72 C° for 2 minutes. Then, 32 cycles were performed as follows; 95 C° for 75 seconds, 55 C° for 75 seconds and 72 C° for 90 seconds. The PCR amplification was completed by a final extension at 72 C° for 6 minutes.

The amplification yields a product 163 bp. Upon cleavage with 5 units of MboII (Boehringer Mannheim) for 16 hours at 37 C° and then, electrophoresed on 3 % agarose and stained with ethidium bromide, and visualized under UV light.

Enzymatic digestion of PCR products of A1298C polymorphism of MTHFR gene using MobII enzyme; MobII digests the 163 bp fragment of the homozygous mutant type CC into 84, 31, 30 and 18 bp fragments. While the wild type AA, the 84 bp fragment is cut into 56 and 28 bp fragments producing 5 fragments of 56, 31, 30, 28 and 18. The heterozygous mutant type CA produced 6 fragments of 84, 56, 31, 30, 28, and 18. All small fragments (31, 30, 28, 18) have run off the gel (**figures 1, 2**)[20].
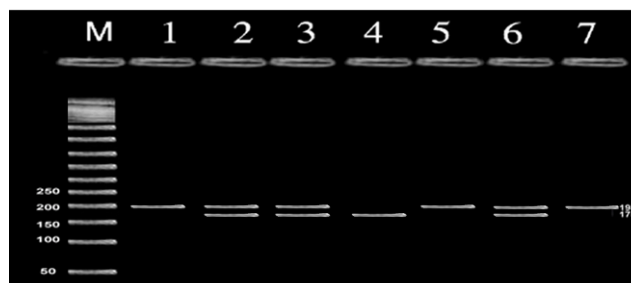

Figure (1): Enzymatic digestion of PCR product of C667T polymorphism of MTHFR gene using HinfI enzyme. HinfI enzyme digests the 198 bp fragment into 175 and 23 bp fragments; the small 23 bp fragment has run off the gel. DNA marker (50 bp), lanes 1,5 and 7 (wild type CC is found which appears at 198 bp), lane 2, 3 and 6 (heterozygous mutated genotype CT which has 198, 175, 23 bp fragments) and lane 4 (homozygous mutated genotype TT is found which has 175, 23 bp fragments).
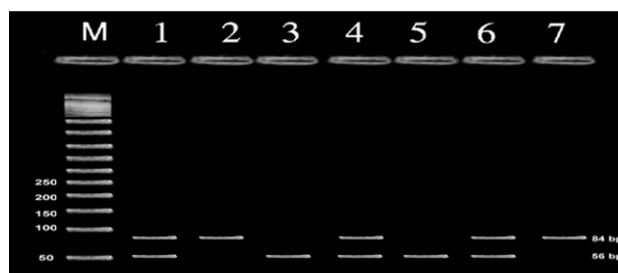
Figure (2): Enzymatic digestion of PCR product of A1298C polymorphism of MTHFR gene using MobII enzyme. MobII enzyme digests the 163 bp fragment of the mutated type CC into 84, 31, 30 and 18 bp fragments, while the wild type AA yields five fragments 56, 31, 30, 28 and 18; the small fragments (18, 28, 30, 31 bp) have run off the gel. Lane M: DNA marker (50 bp), lanes 3, 5 (wild type AA), lanes 1, 4 and 6 (heterozygous mutated genotype AC which has 84, 56, 31, 30, 28 and 18 bp fragments) and lanes 2 and 7 (homozygous mutated genotype CC).

Hypo- Osmotic Swelling (HOS) test for assessment of the functional integrity of the sperm membrane:

One ml of freshly prepared hypo-osmotic medium (0.735 g sodium citrate dihydrate $Na_3C_6H_5O_7$.$2H_2O$ and 1.351 g. fructose in 100 ml distilled water) was added to 0.1ml of liquefied semen and mixed well. The mixture was incubated at 37C º for 30 minutes. The sperm were then examined under phase contrast microscope, and the swelling of sperm tail was identified. The number of swollen cells was counted in duplicate in a total of 100 spermatozoa [17,21].

Preparation of gelatin-covered microslides and gelatinolysis of spermatozoa:

Gelatin-covered slides were prepared by spreading 20μL of 5% gelatin (Merck, Darmstadt, Germany) in distilled water on the slides. The slides were then air-dried, stored at 4°C overnight and fixed and washed in phosphate-buffered saline. Purified spermatozoa were diluted 1:10 in PBS containing 15.7mM α-D-glucose. Semen samples were smeared on prepared slides and incubated in a moist chamber at 37°C for 2 hours. The halo diameter around any 10 spermatozoa shown to be representative of sperm present in the ejaculate was measured in phase contrast with an eyepiece micrometer. The halo formation rate was calculated per slide as the percentage of spermatozoa showing a halo. One hundred spermatozoa were evaluated. An acrosin activity index was calculated by multiplying the halo diameter by the halo formation rate[22].

Measurement of alpha glucosidase activity in seminal plasma:

Measurement of α-glucosidase activity in seminal plasma was done according to the method described by **Guerin et al.**[23] to evaluate the function of the epididymis as the bulk of the activity of α-glucosidase in semen. The kit was supplied from

Ferti Pro N.V. (Industriepark Noord, Beerneme, Belgium).

Separation of S-adenosylmethionine and S-adenosylhomocysteine by high-performance liquid chromatography (HPLC) She et al.[24]

Seminal plasma was mixed 1:2 with 0.4 M $HClO_4$ for 30 minutes and centrifuged at 10000g for 15 min at 4°C; the supernatant was filtered through a 0.2-mm poly-propylene syringe filter (0.4 mm diameter, Whatman, Clifton, NJ, USA). A 20 µl aliquot of the acid extract was applied directly onto the HPLC. SAM and SAH standards (Sigma, St. Louis, MO, USA) were dissolved in water at a concentration of 1 mM and then diluted with 0.4M HClO4 to the final concentration used during HPLC analysis. Aliquots of 20 µl of standard solutions containing 50 –11000 pmol

were injected onto the HPLC. Hewlett Packard HPLC model 1984 B equipped with variable UV detector (Hewlett Packard, 1050 series, USA) adjusted at wavelength 254 nm was used. The separation was done on reversed phase (RP 18 C Lichrosorb, 15 cm - 4.6 mm i.d., Hibar, Merck, Darmastadt, Germany) column. The mobile phase consisted of 40 mM NH4H2PO4, 8 mM 1-heptane sulfonic acid (Sigma, St. Louis, MO, USA), and 18% (v/v) methanol (HPLC grade), pH adjusted to 3.0 with HCl. HPLC analyses were conducted at a flow rate of 0.7 ml/min. HPLC was performed at room temperature. Quantification was based on integration of peak areas and compared to the standard calibration curves of SAM and SAH **(figure 3).** The results are expressed in nmol /L.
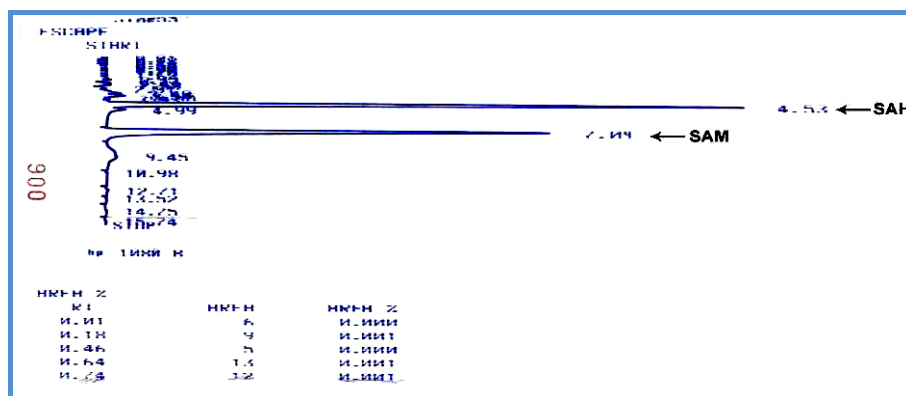

Figure (3): HPLC Chromatogram of standard mixture of SAM and SAH.

Statistical Analysis: Statistical analysis was done by using Med Calc® program version 11.0.1.0[25]. The data were expressed as median, and range. Mann-Whitney test was used as a test of significance for comparison of two groups. P value was considered significant if less than 0.05.

RESULTS

Semen parameters including sperm concentration, grade A motility, grade A+B motility, velocity, linear velocity, linearity index (%) and normal morphology (%) of the different groups are shown in **table (1).**

Table (1): Semen parameters of all the studied groups (range, median):

Parameters	N, n=90	OAT, n=64	AZ n=75
Concentration (million /ml)	7.6 (2.5-18.6)	68.30 (10.8-96.8)	-
Grade A motility (%)	6.5 (0.00-20)	41.9 (25.3-60)	-
Grade A+B motility (%)	16.5 (0.00-41)	54.00 (31.5-69.1)	-
Velocity (µm/sec)	21.00 (1.2-74.5)	73.4 (55-95.4)	-
Linear velocity (µm/Sec)	11.3 (0.2-52.6)	56.3 (41.5-75.5)	-
Linearity index (%)	55.9 (15.8-97.6)	77.8 (60.00-95.6)	-
Normal morphology (%)	3.00 (0.00-10.00)	43.9 (26.6-66.00)	-

N = Normozoospermia   OAT = Oligoasthenoteratozoospermia
A z = Azoospermia   n = number of cases

Table (2) showed alpha-glucosidase activity**,** acrosine activity index, HOST (%), SAM and SAM/SAH ratio to be significantly decreased in patients groups compared with the control group. However, SAH was significantly increased in OAT and AZ groups compared to the control group

Table (2): Alpha-glucosidase activity, acrosin activity index, HOST (%), SAM, SAH and SAM/SAH ratio in all studied groups (range, median)

Parameter	N , n=90	OAT, n=64	AZ , n=75	p-value
α-glucosidase Activity (U/ml)	61.1 (31.00-90.7)	28.5 (18.4-45.3)	19.8 (12.6-31.5)	p_1<0.0001, p_2<0.0001 p_3<0.0001
Acrosin activity index	11.6 (7.5-19.4)	3.7 (0.6-7.8)	-	p_1<0.0001
HOST (%)	86 (72-94)	44 (6-79)	-	p_1<0.0001
SAM (nmol/l)	100.2 (76.8-160)	81.02 (60.1-108.6)	63.7 (44.8-91.7)	p_1<0.0001, p_2<0.0001 p_3<0.0001
SAH (nmol/l)	25.4 (9.4-38.04)	36.5 (23.6-44.2)	43.08 (31.4-52.5	p_1<0.0001, p_2<0.0001 p_3<0.0001
SAM/SAH ratio	4(2.1-9.8)	2.2(1.5-4.4)	1.5(0.9-2.8)	p_1<0.0001, p_2<0.0001 p_3<0.0001

N = Normozoospermia, OAT = Oligoasthenoteratozoospermia, AZ = Azoospermia
p_1=significance between N and OAT, p_2= significance between N and AZ
p_3= significance between OAT and AZ, n = number of cases

The result of PCR determination of **MTHR C677T**   gene revealed that **MTHR 677CC** genotype frequency was significantly higher in N compared with OAT and AZ groups. However, **MTHR 677TT genotype** frequency was found to be significantly higher in OAT and AZ groups compared with the control**.** Also, **MTHR 677TT** genotype frequency was found to be significantly higher in AZ than OAT group **(table 3)**.

Table (3): Frequency of MTHR C677T genotypes and alleles among the studied groups :

MTHR C677T Genotypes and alleles	N n=90	OAT n=64	AZ n=75	p-value
CC n=96	41 (45.5%)	35 (59.7%)	20 (26.7%)	P_1=0.07 P_2=0.02 P_3=0.0002
CT n=87	44 (48.9%)	20 (31.2%)	23 (30.7%)	P_1=0.03 P_2=0.01 P_3=0.9
TT n=46	5 (5.6%)	9 (14.1%)	32 (42.7%)	P_1=0.1 P_2 <0.0001 P_3=0.0005
C allele	126 (70%)	70 (70.3%)	63 (42%)	P_1=0.9 P_2=0.0007 P_3=0.001
T allele	54 (30%)	38 (29.7%)	87 (58%)	P_1 <0.0001 P_2=0.17 P_3=0.001

N = Normozoospermia, OAT = Oligoasthenoteratozoospermia, AZ = Azoospermia
n = number of cases p_1=significance between N and OAT p_2= significance between N and AZ, p_3= significance between OAT and AZ

The result of PCR determination of **MTHR A1298C** gene revealed that **MTHR 1298AA** genotype frequency was significantly higher in N compared with OAT and AZ groups. However, **MTHR 1298CC** genotype frequency was found to be significantly higher in OAT and AZ groups compared with the control. Also, **MTHR 1298CC** genotype frequency was found to be significantly higher in AZ than OAT group **(table 4)**.

Table (4): Frequency of MTHR A1298C genotypes and alleles among the studied groups:

MTHR C677T Genotypes and alleles	N n=90	OAT n=64	AZ n=75	p-value
AA n=66	39 (43.3%)	15 (23.4%)	12 (16%)	P_1=0.01 P_2=0.0003 P_3=0.37
AC n=135	48 (35.3%)	40 (62.5%)	47 (62.7%)	P_1=0.001 P_2=0.0008 P_3=0.87
CC n=28	3 (3.3%)	9 (14.1%)	16 (21.3%)	P_1=0.03 P_2=0.0008 P_3=0.3
A allele	126 (70%)	70 (54.7%)	71 (47.3%)	P_1=0.07 P_2=0.005 P_3=0.4
C allele	54 (30%)	58 (45.3%)	97 (52.7%)	P_1=0.07 P_2=0.005 P_3=0.48

N = Normozoospermia, OAT = Oligoasthenoteratozoospermia, AZ = Azoospermia
n = number of cases, p_1=significance between N and OAT, p_2= significance between N and AZ, p_3= significance between OAT and AZ

Table (5) showed that sperm concentration, grade A motility, grade A+B motility, velocity, linear velocity, and normal morphology (%) were significantly decreased in individuals with MTHR 677TT genotype than MTHR 677CT and MTHR 677CC.

Table (5): Semen parameters in different MTHR C677T genotypes (Median and Range):

Parameters	CC (n=96)	CT (n=87)	TT (n=46)	p-value
Grade A motility (%)	39.7 (0.00-60)	30.4 (0.00-60)	11.5 (0.00-42.2)	$p_1 = 0.06$ $p_2 = 0.2$ $p_3 = 0.006$
Grade A+B motility (%)	50.7 (0.00-66)	41.2 (0.00-69.1)	27.5 (0.00-61)	$P_1 = 0.07$ $p_2 = 0.28$ $p_3 = 0.02$
Velocity (µm/Sec)	68 (1.4-95.4)	61 (1.2-95.4)	26 (2.0-86)	$p_1 = 0.13$ $p_2 = 0.01$ $p_3 = 0.12$
Linear velocity (µm/Sec)	50.7 (0.2-66)	47.5 (0.2-73.5)	27.5 (0.7-61.5)	$p_1 = 0.19$ $p_2 = 0.02$ $p_3 = 0.25$
Linearity index (%)	76.4 (18.8-90.8)	74.7 (17.8-90.6)	67.7 (36.9-85.3)	$p_1 = 0.16$ $p_2 = 0.04$ $p_3 = 0.25$
Normal Morphology (%)	38.8 (0.00-64)	33.1 (0.00-64)	6.0 (0.00-44.2)	$p_1 = 0.3$ $p_2 = 0.005$ $p_3 = 0.06$

p_1=significance between CC and CT, p_2= significance between CC and TT
p_3= significance between CT and TT

Also, **table (6)** showed alpha-glucosidase activity**,** acrosine activity index , HOST (%) ,SAM and SAM/SAH ratio to be significantly decreased in individuals with MTHR 677TT genotype than MTHR 677CT and MTHR 677CC. However SAH level was increased in individuals with MTHR 677TT genotype than MTHR 677CT and MTHR 677CC.

Table (6): Alpha-glucosidase activity, acrosin activity index, HOST (%), SAM, SAH and SAM/SAH ratio different MTHR C677T   genotypes (Median and Range):

Parameters	CC (n=96)	CT (n=87)	TT (n=46)	P
Acrosin activity index	9.07 (0.7-19.4)	10.1 (0.6-14.1)	4.8 (0.6-14.3)	p_1= 0.8 p_2= 0.03 p_3= 0.04
HOST(%)	79 (9-93)	75 (12-94)	51 (6-91)	p_1 = 0.11 p_2 = 0.25 p_3 = 0.09
Alpha-glucosidase activity(U/ml.)	38.4 (15-99.7)	33.9 (12.6-88)	21 (13.7-84.4)	p_1= 0.9 p_2<0.0001 p_3<0.0001
SAM (nmol/l)	89.5 (47.9-160)	85 (44.8-144.7)	74 (45.3-18.6)	p_1= 0.95 p_2= 0.0004 p_3= 0.0008
SAH (nmol/l)	31.6 (9.5-49.1)	32.1 (11.8-52.5)	40.8 (11.3-52.5)	p_1= 0.7 p_2< 0.0001 p_3< 0.0001
SAM/SAH ratio	2.7 (1-9.8)	2.9 (0.9-8.9)	1.8 (0.95-8.8)	p_1= 0.8 p_2< 0.0001 p_3< 0.0001

p_1=significance between CC and CT p_2= significance between CC and TT
p_3= significance between CT and TT

Table (7) showed that sperm concentration, grade A motility, grade A+B motility, velocity, linear velocity, and normal morphology (%) were significantly decreased in patients with MTHR 1298CC genotype than MTHR 1298AC   and MTHR 1298AA.

Table (7): Semen parameters in different MTHR A1298C genotypes (Median and Range):

Parameters	AA (n=66)	AC (n=135)	CC (n=28)	p-value
Grade A motility (%)	39.6 (0.00-60)	30.9 (0.00-60)	9.5 (0.00-37.2)	p_1 = 0.03 p_2 = 0.001 p_3 = 0.02
Grade A+B motility (%)	51.7 (0.00-67)	41.2 (0.00-69.1)	27 (0.00-57.4)	P_1 = 0.01 p_2 = 0.002 p_3 = 0.1
Velocity (μm/Sec)	68.1 (1.2-95.4)	59 (1.4-95.4)	24 (2.1-86)	p_1 = 0.09 p_2 = 0.005 p_3 =0.06
Linear velocity (μm/Sec)	52.3 (0.2-75.5)	48.5 (0.2-73.5)	13.5 (0.7-64.5)	p_1 = 0.05 p_2 = 0.006 p_3 =0.08
Linearity index (%)	77.4 (18.8-90.8)	72.7 (15.8-97.6)	61.7 (36.9-85.3)	p_1 = 0.17 p_2 = 0.01 p_3 = 0.04
Normal Morphology (%)	40.8 (0.00-66)	32.1 (0.00-64)	4 (0.00-45.2)	p_1 = 0.003 p_2 = 0.01 p_3 = 0.06

p_1=significance between AA and AC p_2= significance between AA and CC
p_3= significance between AC and CC

Also, **table (8**) showed alpha-glucosidase activity**,** acrosine activity index , HOST (%), SAM and SAM/SAH ratio to be significantly decreased in individuals with MTHR 1298CC genotype than MTHR 1298AC and MTHR 1298AA However SAH level was increased in individuals with MTHR 1298CC genotype than MTHR 1298AC and MTHR 1298AA.

Table (8): Alpha-glucosidase activity, acrosin activity index, HOST (%), SAM, SAH and SAM/SAH ratio different MTHR A1298C   genotypes (Median and Range):

Parameters	CC (n=66)	CT (n=135)	TT (n=28)	P
Acrosin activity index	10.4 (0.7-27.25)	9.36 (0.6-19.5)	4.8 (0.6-14.3)	p_1= 0.11 p_2= 0.02 p_3= 0.08
HOST(%)	79 (9-94)	74 (12-94)	46 (6-91)	p_1 = 0.11 p_2 = 0.01 p_3 = 0.06
Alpha-glucosidase activity(U/ml.)	48.8 (12.6-90.7)	30.7 (12.6-88)	22.4 (13.7-84.4)	p_1= 0.002 p_2=0.0001 p_3<0.0001
SAM (nmol/l)	90.5 (44.9-160)	81.7 (44.8-144.7)	81.4 (45.3-108.6)	p_1= 0.01 p_2= 0.02 p_3= 0.96
SAH (nmol/l)	30.6 (9.5-45.1)	34.1 (11.8-52.5)	38.8 (24.3-52.5)	p_1 = 0.004 p_2< 0.0001 p_3< 0.0001
SAM/SAH ratio	3.1 (1-9)	2.7 (0.9-9.8)	2.0 (0.9-3.8)	p_1= 0.0007 p_2=0.0006 p_3=0.3

p_1=significance between CC and CT p_2= significance between CC and TT
p_3= significance between CT and TT

DISCUSSION

DNA methylation is an important epigenetic feature of DNA that plays a pivotal role in gene expression regulation during spermatogenesis. The enzyme methylenetetrahydrofolate reductase (MTHFR) catalyses the formation of folate intermediates that are vital for DNA synthesis and methylation reactions [26].

The two common mutations in the MTHFR gene, the cytosine-to-thymine substitution at base 677 (C677T) and adenine-to-cytosine substitution at base 1298 (A1298C), creating a thermolabile enzyme variant and somewhat reduced enzyme activity in vitro. These two mutations predispose to hyperhomocysteinemia, especially in folate-deficient subjects[27]. Methylenetetrahydrofolate reductase (MTHFR) plays an important role in the process of DNA, RNA and protein metabolism, and is closely related with spermatogenesis. The polymorphisms of MTHFR C677T and A1298C may have a close relationship with male infertility [28]..

The current study, also, suggests the existence of a potential link between 677TT and 1298CC genotypes and alterations in both spermatozoa production and sperm motility. A few previous studies have evaluated the association of MTHFR C677T polymorphism in infertile patients from Germany, Netherlands,

Italy, India, South Korea and China[12,29-32]. Some of them[12,29,32] have reported an association between this polymorphism in the MTHFR gene and male infertility. **Varinderpal et al.**[2] reported no association between the A1298C SNP and male infertility in an Indian study group. Also, **Farcos et al.**[33] found that there is no significant association of SNP A1298C in the MTHFR gene with azoospermia or oligozoospermia in Romanian population. The discrepancy of the results between the different studies can be attributed to the variability of the number of patients studied or/and to the ethnic differences regarding the distribution of this pattern of 677 C→T and 1298 A→C polymorphisms **Chandy et al.**[34].

Several studies have now reported that spermatozoa from infertile men are more likely to express aberrant DNA methylation patterns [35-37].

In the current study, the significantly decreased SAM, SAM/SAH ratio and acrosine activity index in infertile men and the positive correlation between SAM/SAH ratio and sperm parameters were in harmony with the results of **Singh et al.**[12] **and Paracchini et al.**[13]. Extreme abnormalities in homocysteine metabolism may be associated with alterations in sperm DNA methylation, but minor elevations in homocystine do not affect on sperm DNA methylation status [38].

The present study reveals that the levels of S-adenosyl homocysteine were dependent on MTHFR C677T genotypes. S-adenosyl homocysteine

in patients with TT genotype (40.8 nmol/l) and CT genotype subjects (32.1nmol/l) were significantly higher as compared to patients with CC genotype (31.6 nmol/l, P<0.05), This is in accordance with the findings of other workers [27,39]. These results indicate a strong graded association between S-adenosyl homocysteine and infertility risk in TT and CT genotypes. In this study, also, the present study reveals that the median values of S-adenosyl homocysteine were dependent on MTHFR A1298C genotypes there was significant difference between the level of S-adenosyl homocysteine in AA genotype (30.6 nmol/l), AC genotype (34.1 nmol/l) and CC genotype (38.8 nmol/l, P<0.05). This is in agreement with the findings of other workers [27]. However, others showed that there was no significant difference in mean values of S-adenosyl homocysteine and MTHFR A1298C genotypes [40]

MTHFR C677T results in decreased formation of active folate, methyltetrahydrofolate[41] which in turn leads to an increase in plasma total homocysteine concentrations. The MTHFR C677T polymorphism is considered the single most important genetic determinant of plasma homocysteine [42].

It has already been shown that sperm concentration is increased by folic acid and zinc sulphate treatment. Also, in the cause of altered folate status due to reduced MTHFR enzyme activity, epigenetic alterations in DNA must be taken into account as important etiological factor [30].

Considering the fact that folate deficiency has been shown to reduce the proliferation of various cell types

[43] and also that it is already established that folate intake is very important for male infertility, future studies need to focus on the relation between idiopathic cases of infertility, genetic risk factors and the nutritional status of subjects; dietary habits which are particular in the country were the study is conducted influence plasmatic levels of homocysteine and folate.

CONCLUSION

The single nucleotide polymorphism in the MTHFR C677T and A1298C were associated with abnormal sperm function, morphology and motility. The increased SAH associated with 677TT and 1298CC genotypes might have a role in the pathogenesis of male infertility. It may be necessary to enlarge the study groups in order to obtain more significant conclusions and to evaluate other polymorphisms in genes that code for key enzymes in the folate and homocysteine metabolism, for being able to interpret the eventual complex gene-gene interactions with possible implications in the studied pathology.

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تعدد الأشكال الجينية لجين الميثيلين تتراهيدروفوليت ريدكتيز و اختلال أيض المثيونين في عقم الرجال

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هدف البحث: يهدف هذا البحث إلى دراسة النمط الجينى لجين الميثيلين تتراهيدروفوليت ريدكتيز بنوعيه (سيتوزين ٦٧٧ ثايمين) و (أدنين ١٢٩٨ سيتوزين) والاس ادينوزايل مثيونين والاس ادينوزايل هوموسيستايين فى حالات عقم الرجال.

طريقة البحث: أجريت هذه الدراسة على ١٩٠ عينة سائل منوى من الرجال المترددين على العيادة الخارجية لأمراض الذكورة فى مستشفى المنصورة الجامعى ، حيث أخذت عينات السائل المنوى وقسمت كالتالى: ٩٠عينة طبيعية كمجموعة ضابطة و ٦٤ عينة ذات حيوانات منوية قليلة العدد ضعيفة الحركة مشوهة الشكل ، و ٧٥ عينة عديمة الحيوانات المنوية . وقد تم تحليل حركة وعدد الحيوانات المنوية فى العينات باستخدام جهازالأوتوسبيرم وتم اختبار نشاط الأكروزين تبعا لتوصيات منظمة الصحة العالمية. وقد أخذت عينات دم من أجل استخلاص الحامض النووي (دي إن إيه) واستخدامه في تحديد النمط الجينى لجين الميثيلين تتراهيدروفوليت ريدكتيز بنوعيه (سيتوزين ٦٧٧ ثايمين) و (أدنين ١٢٩٨ سيتوزين) بواسطة تفاعل البلمرة المتكرر ثم التحليل باستخدام الإنزيمات الحصرية.

نتائج البحث: ـ وقد أوضحت نتائج الدراسة لجين الميثيلين تتراهيدروفوليت ريدكتيز بنوعيه (سيتوزين ٦٧٧ ثايمين) و (أدنين ١٢٩٨ سيتوزين) بواسطة تفاعل البلمرة المتكرر ثم التحليل باستخدام الإنزيمات الحصرية.

ـ وجود٦.٥% من TT٦٧٧ فى عينات السائل المنوى الطبيعى ـ و١٤.١% فى العينات ذات حيوانات منوية قليلة العدد ضعيفة الحركة مشوهة الشكل ، ٤٢.٧% فى العينات عديمة الحيوانات المنوية ، وجود ٣.٣% من CC١٢٩٨ فى عينات السائل المنوى الطبيعى ١٤.١% فى العينات ذات حيوانات منوية قليلة العدد ضعيفة الحركة مشوهة الشكل ، و٢١.٣% فى العينات عديمة الحيوانات المنوية ،

وقد أظهرت هذه الدراسة وجود علاقة ارتباط إيجابى ذات دلالة إحصائية بين الاس ادينوزايل هوموسيستايين من ناحية ، وبين النمط الجينى TT٦٧٧ و CC١٢٩٨ لجين الميثيلين تتراهيدروفوليت ريدكتيز. وأيضا وجود نقص ذا دلالة إحصائية فى معامل نشاط الأكروزين ، وألفا جلوكوسيداز وحركة الحيوان المنوى (الدرجة أ ، ب)، والسرعة الخطية فى المرضى ذوي النمط الجيني TT٦٧٧ و CC١٢٩٨ لجين الميثيلين تتراهيدروفوليت ريدكتيز.

يمكن الاستنتاج من هذا البحث أن النمط الجينى TT٦٧٧ و CC١٢٩٨ لجين الميثيلين تتراهيدروفوليت ريدكتيز مرتبط بزيادة الاس ادينوزايل هوموسيستايين فى عقم الرجال ومن ثم يمكن تقديم طرق علاجية جديدة لحالات العقم فى الرجال عن طريق التحكم فى زيادة حمض الفوليك وتقليل الاس ادينوزايل هوموسيستايين.