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Research Article

ANALYSIS OF METHYLENETETRAHYDROFOLATE REDUCTASE (MTHFR) C677T AND A1298C POLYMORPHISMS ON MALE INFERTILITY

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ABSTRACT
DNA methylation is an important epigenetic feature of DNA that plays n pivotal role in regulation of gene express ion during spermntogenesis. C677T and A 1298C Mutations are known ns causes of low MTHFR enzymatic activity, so polymorphic variants in the MTHFR gene may be associated with male infertility in some populations. To assess the relation between the C677T and A 1298C polymorphisms and male infertility in Iran, we studied the C677T mutation in 303 infertile men and 300 fel1i le men as control and the A 1298C mutation in 131 infertile men and 130 fertile men as control. Genomic DNA was extracted by salting out procedure. Detection of the MTHFR C677T and A 1298C mutation was performed by PCR-RFLP analysis. The frequencies of Cc. CT, and IT genotypes in patients were 53.7%, 36%, 10.3% and in controls were 49.835%. 40.925%, 9.24% respectively (p=0.458). The frequencies of AA, AC, and CC genotypes in patients were 41.98%. 40.46%. 17.56% and in controls were 35.385%, 55.385%, 9.23% respectively (P=O.066). Our findings suggest that there is no significant association of C677T and A 1298C in the MTHFR gene with male infertility in assessed Iranian a subpopulation. There is significant association of Heterozygosity in both polymorphisms, indicating that these polymorphism together may play a role in infertility (P=0.035).

Keywords: *MTHFR, Gene Polymorphism, Male Infertility*

INTRODUCTION
Infertility refers to the inability of a couple to conceive after one year of sexual intercourse without using any contraceptive method (Alberto *et al.,* 2006). Infertility is a global problem, and about one through seven couples affected by infertility or reducing fertility (Botto and Yang, 2000). Statistical analysis showed that the prevalence of infertility in developing societies is increasing, so this is a common and concerning problem for the couples.

According to a study in 1987 in several centers under the auspices of WHO (World Health Organization), in 20% of infertile couples only men factor, about 38% only women factor and 27% both male and female factors were involved together.

The remaining 15% is still a cause that has not been found. These are called idiopathic infertility, mean that the couples have no problem with regard to existing experiments, but for unknown reasons, and could not conceive children. Idiopathic infertility can be caused by factors such as chronic diseases, malnutrition, environmental factors or genetic disorders that they have not been identified yet (Chango *et al.,* 2000).

Although, the causes of infertility are numerous and each couple must be fully evaluated, but several studies have examined the role of male infertility.

In other words, evaluation and treatment of patients in the course of resolving infertile men problems is very important (Brattstrom, 1998).

Naturally, if any of the factors of maturity, number, shape and the ability and suitable movement of the sperm has abnormalities, the chance of fertilization is low and the man is infertile.

Infertility is a heterogeneous problem in which multiple genetic and environmental factors are involved. Male infertility is related with several disorders, including disorders in the number, motility and morphology of sperm (Engbersen *et al.,* 1995; Foresta *et al.,* 2002; Ferlin and Foresta, 2005).

The standard semen parameters according to World Health Organization are presented in Table 1:

Table 1: The Standard Semen Parameters According to World Health Organization

Volume	**2 ml>**
Acidity (pH)	7/2-7/8
Sperm Concentration	20×106/ml Spermatozoa
Total Sperm Count	40 × 106 Spermatozoa in the Ejaculation
Motility	50% < Move Forward
Morphology	30% < Standard Forms

In general, the causes of male infertility could be genetic, anatomic, hormonal, inflammatory, immunologic and mechanical damages. Many factors, including erectile dysfunction, premature ejaculation, hormonal problems, obesity and sexually abnormal cells, infection and even the necessity of surgery which lead to infertility, all may be trivial factor related to the genetic factors with particular importance in the infertility (Ebisch, 2003; Ezeh, 2000). The most common non-genetic causes of male infertility include Haypognadysm, structural abnormalities of the genital, genital infections, surgery of the scrotum, varicocele, chronic diseases, drugs and chemicals materials. Gene defects and chromosomal abnormalities can impair spermatogenesis process. Infertility and its association with genetic and chromosomal factor is very complex (Anonymous, 1998). Gene mutations, chromosomal aberrations, abnormalities in sperm chromatin network and its maturity are some factors that can disrupt the formation of sex cells and ultimately impair fertility. The occurrences of certain events, such as pre mature concentration of genetic material of the sperm into the oocyte are also known causes of infertility (Bhasin, 1994; Bezold and Lange, 2001). As a result, the most important step in determining the cause of the disease is identifying the genetic factors contributing to infertility (Bailey and Gregory, 1999).

C677T Polymorphism in the MTHFR Gene

One of common polymorphism in the MTHFR gene is C677T which causes instability and reducing the normal and thermal of MTHFR enzyme. This polymorphism alters the alanine amino acid to valine and at the position polypeptide 222 is derived. In a study by Goyette *et al.*, 1995, 15% of this alleles, were mutant homozygous (T / T). The enzymes in these people were heat-unstable, and showed severe reduction in activity than heated C / C. Reducing or stopping the MTHFR enzyme activity decrease the enzyme product (methyltetrahydrofolate) and increase substrate (5, 10, methylene tetrahydrofolate). Although increasing 5, 10- methylene tetrahydrofolate, provides favorable conditions for the synthesis of DNA and nucleotides, but decreasing 5 - methyltetrahydrofolate, as the methyl donor cell, endanger biosynthesis of methionine from homocysteine. Methionine, which is reducing with increasing of homocysteine, reduce cellular metabolism and also may increase the toxicity of cells (Cravo *et al.*, 1996). The first study was conducted on patients in relation to the MTHFR genetic defect that their plasma HPLC chromatography, had approved high levels of homocysteine and symptoms such as cerebrovascular and peripheral vessel damage and heart problems was detected. Patients with the lowest level of enzyme activity (0 to 3% of control) show the first symptoms in the first year of life, while those with enzymatic activity show these symptoms mainly in the second decade of life. C677T polymorphism reduces the activity of the enzyme to 37 C ° and increases its thermal instability 46°C. This change has been described with directed mutation technique in healthy DNA and its expression in prokaryotic systems (Dallaire and Huret, 2005). When the bacteria extract heated to 46C ° and for five minutes, the mutant enzymes have less activity compared to wild-type as well as heated. While homozygous mutant 40 T / T have activity to 50% of the C / C samples and activities of mutant C / T in both state 37C ° and 46 C º , the natural state is almost 50% (Farcas *et al.*, 2009; Ferlin *et al.*, 2006). Plasma homocysteine and methionine concentrations measured in different patients have shown that the average plasma homocysteine mutants have 677 more than normal. According to numerous studies on the polymorphism C / T, is clear that there are differences in different populations. Analysis of white and Asian has shown that 12 percent are homozygous T / T and more than 50% are heterozygous. This figure is too high relative to the frequency of allelic African population (Ferlin *et al.*, 2005). According to studies carried out in Italy (Chango *et al.*, 2000), France (Alberto *et al.*,2006), Spain (Brackcleer and Dao, 1991),

Canada (Brown, 2001), China (Dhillon *et al.*, 2007), Japan (15) and Norway (Dohle *et al.*, 2002), was about 8.23, 2 / 18, 8/15, 3/15, 3/14, 2/10 and 8 percent respectively.

A1298C Polymorphisms in the MTHFR Gene

1298 polymorphism in MTHFR gene was found for the first time in 1999, during the study of ovarian cancer. This polymorphism in exon 7 led to the replacement of glutamine instead of alanine in a polypeptide chain. Substitution of Glutamine, instead valine, mildly reduce MTHFR enzyme activity in it, it does not affect on protein thermal instability (Dhillon *et al.*, 2007). In 1298 Polymorphism, reduction of the MTHFR enzyme activity was seen in samples of homozygous C / C and heterozygous A / C does not create a significant reduction in enzyme activity (Ferlin *et al.*, 2005). A wide range study does not carried out on the allelic prevalence of A1298C polymorphism. Studies show that homozygous genotype A1298C in control, was almost 9% in Canada (Ebisch *et al.*, 2003) and the Netherlands (Bhasin *et al.*, 1994), respectively. In a study of people with high homocysteine, the frequency of allelic C was measured at 34 percent (Ezeh, 2000).

Male infertility is an issue that currently is highly regarded and has been studied. In the past, the only cause of infertility was assumed due to problems of women. But in recent decades, the role of men is known in such disease.

Several Genetic and environmental factors involved in male infertility. That is why; despite the efforts made, identifying a number of effective factors in the disease and finding solutions to resolve the problem is still a global issue. Several genetic factors such as polymorphisms in some genes may be involved in this disease (Botto and Yang, 2000).

On the basis of a positive relationship between serum folate concentration with sperm density, there is sperm progressive motility and normal morphology, so probably folate pathway is important in male fertility. Methylene tetrahydrofolate reductase (MTHFR) is a key enzyme in folate metabolism, DNA base synthesis and reactions of Ramtylasyvn.

Polymorphisms of this gene by reducing MTHFR enzyme activity and subsequently creating defects in folate cycle could have a role in male infertility. Different population studies indicate that polymorphic variants of MTHFR gene such as C677T and A1298Cb decrease sperm count and male infertility in some populations (Anonymous, 1998). Given the importance of these two polymorphisms in the MTHFR gene, in this study, the importance of two polymorphisms of A1298C and C677T will be analyzed in infertility of Iranian men.

MATERIALS AND METHODS

Blood Samples

Blood samples studied in this research is related to male infertility (infertility refer to the inability of a couple to conceive after one year of sexual intercourse without using any contraceptive method) referred to the infertility specialists in Sarim Hospital in Tehran. These patients have been referred to this center from Tehran province and other cities. After visiting the patients and necessary examinations and tests to determine the cause of infertility, including hormonal disorders, reproductive system problems, infectious diseases, genetic disorders, etc., 300 men were selected for reviewing in this research. 303 fertile men (at least with two children) were studied as a control. Then from all these people, 2 ml of blood were taken and transferred to Falcon containing EDTA anticoagulant.

DNA Extraction from the Blood by Salting Out Method

Blood is suitable tissue for the preparation of template DNA and different biological tests particularly PCR. The Genomic DNA must be extracted from white blood cells. There are several ways to extract. The Salting out method wad used because of cost-effective, easy and quality of DNA In this research. After concentration, extracted DNA was kept for the next review at -4 C^{o}.

PCR Method

PCR reactions carried out in micro tubes 0/2 ml. The final volume was 25 micro litters by adding requirements of PCR in any micro tubes, and then PCR reaction was performed after optimizing in the Techne. Table 2, present the concentration of the components for PCR.

Table 2: Required Materials of PCR for C677T and A1298C Polymorphisms

A1298C	C677T	Materials
F:0/5	F:1	Primer: 10 pmol/µl (µl)
R:0/5	R:1	Primer: 10 pmol/µl (µl)
2	2	MgCl2 (µl)
2/5	2/5	PCR buffer 10X (µl)
2	1	dNTP 10Mm (µl)
0/25	0/5	Taq DNA Polymerase: 5u/ µl (µl)
0/5	0/75	Genomic DNA (µl)
16/75	16/25	H2O (µl)
25	25	The final volume (µl)

After preparing the required materials of PCR according to Table 2, for the MTHFR gene reproduction in order to evaluate A1298C and C677Tpolymorphisms and determine the thermal condition of C677T polymorphism in Table 3 and Table for A1298C polymorphism. The thermal program for standard PCR is according to Tables 3 and 4.

Table 3: PCR conditions for C677T Polymorphism

Starter	Denaturation Time and Temperature	30 Cycle			Final Elongation Time and Temperature
		Denaturation Time and Temperature	Connection Time and Temperature	Elongation Time and Temperature	
C677T	5 minute 94C º	30 second 94C º	40 second 67C º	40 second 72C º	5 minute 72C º

Table 4: Conditions of PCR for A1298C Polymorphism

Starter	Denaturation Time and Temperature	32 Cycle			Final Elongation Time and Temperature
		Denaturation Time and Temperature	Connection Time and Temperature	Elongation Time and Temperature	
C677T	5 minute 94C º	30 second 94C º	40 second 67C º	40 second 72C º	5 minute 72C º

Evaluation of C677T Polymorphism MTHFR Gene

For determining of C677T polymorphism in the MTHFR gene, first the segment of nucleotide 198 bp gene replicated by PCR. This segment was placed in reaction of RFLP under HinfI enzyme. This enzyme is a restrictive endonuclease that after identifying its own nucleotide sequence, cutting it off. The C677C homozygote's person without this nucleotides sequence set at codon 4 MTHFR gene and under the enzyme will not be cut. As shown schematically in Figure 1, C677T polymorphism, leads to the identification of the enzymes HinfI and cut the DNA in this place. Therefore, homozygous genotype C677C on gel shows only one band of 198 base pair (bp). Heterozygous genotype for C677T polymorphism after enzymatic resection create three bands 198, 175 and 23 basic pair in which, the band of 23 basic pair due to its small size will not be visible on agarose gel. Homozygous genotype C677T (mutant) is also due to the resection in both alleles leads to two bands of 175 and 23 basic pairs.

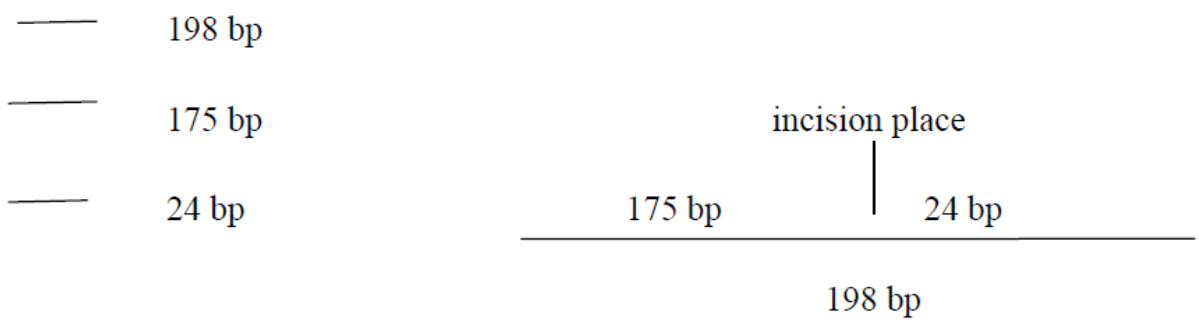

Figure 1: A Schematic Effect on the Enzyme HinfIenzyme on Nucleotide 198 PCR Pairon 677MTHFR Genes

Analysis of A1298C Polymorphism MTHFR Gene

The PCR product for studying this polymorphism is a segment with nucleotide 241 bp. This segment was subjected to MboII restrictive enzyme. One A1298A homozygous person, have no incision place in the fragment of 241bp, so the resulting mutant genotype A1298C due to the acquisition of the incision because of mutation, after RFLP, three 241 bp, 204, and 37 bp and in C1298C genotypes two segment of 204 and 37bp are obtained. However, the bp 37 band due to the small size will not be visible in the gel. The incision place of MboII enzymes is shown schematically in Figure 2.

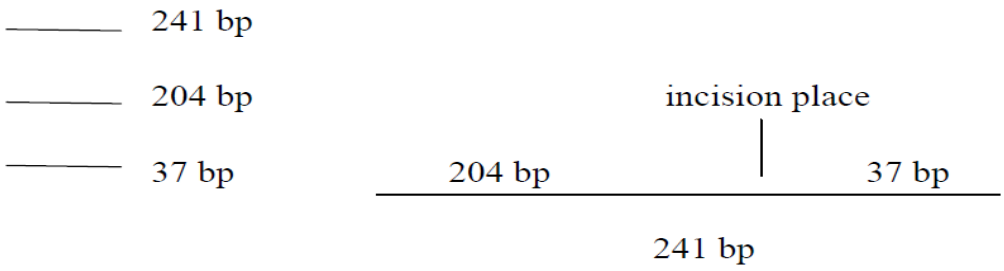

Figure 2: A Schematic of MboII Enzymes Effect on the PCR Nucleotides 241 Pairs of in the 1298 MTHFR Gene

Statistical Analyses

For the results of laboratory research, using statistical parameters are required. The study results were evaluated by using Fisher's Exact Test, based on SPSS software (version 18). The results of each polymorphism separately were compared between patient and control groups.

And also the combined effect of these polymorphisms are obtained by this test, and P-value was calculated in all cases.

A P-value is realized the error occurred during a trial, if the amount obtained from this factor is less than or equal to 05/0, the results in a statistically research would be significant. In other words, a factor under study are involved in the incidence, otherwise there will be no connection between them. In study of 130 patients, 46 patient in control group were AA, 72 patients were heterozygous AC and 12 patient were homozygous CC.

Out of 131 patients 55patient were AA, 53 patients were heterozygous AC and 23 patient were homozygous CC. The results of the statistical analysis of A1298C polymorphism, comparison of the control group with patients is shown in Table 5 and Figure 3.

Table 5: Frequency and Percentage of Genotypes AA, AC and CC about A1298C Polymorphism in Control Group and Patient

Frequency of Control Group (Relative Percentage)	Frequency of Patient Group (Relative Percentage)	A1298C
46 (%35/385)	55 (%41/98)	AA
72 (%55/385)	53 (%40/46)	AC
12 (%9/23)	23(%17/56)	CC
130 (%100)	131 (%100)	TOTAL

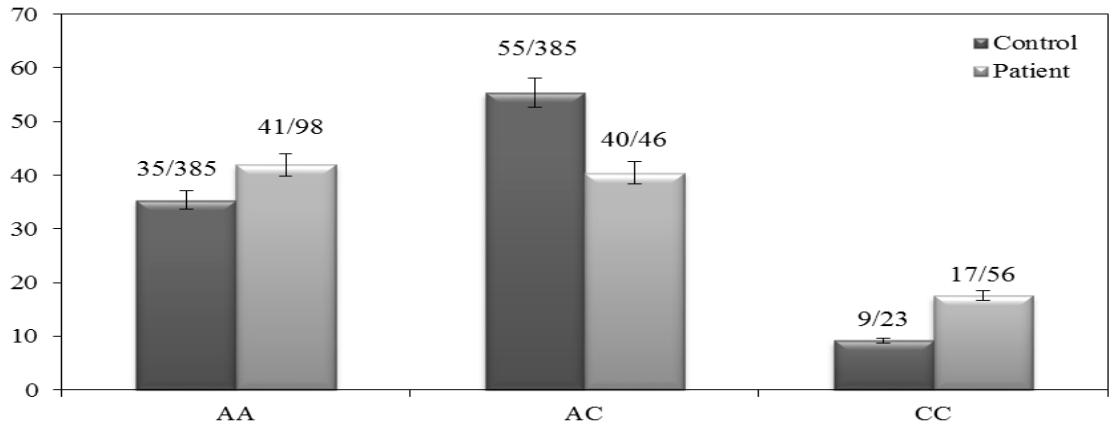

Figure 3: Comparison of Genotypes AA, AC, and CC about A1298C Polymorphism in the MTHFR Gene between the Patient and Control Groups

In analyzing of A1298C polymorphism in the analysis of the results did not show a significant difference between the control group and patients. A P-value is greater than 05/0 to assume that the lack of correlation between the two variables is not rejected. So, polymorphism A1298C in our population has no effect on male infertility.

Analysis of Genotype Frequencies of C677T and A1298C

After analyzing polymorphisms separately, different genotypes of both polymorphisms were also noted. The results are shown in Table 6.

Table 6: Evaluation of the Combined Genotype Frequencies of C677T and A1298C

Variable	Control Group		Patient Group		P-Value
	Relative Percentage	Frequency	Relative Percentage	Frequency	
C677T/A1298C	0/12	36	0/066	20	0/035
C677C/C1298C	0/039	12	0/076	23	0/057
C677C/A1298A	0/59	18	0/57	17	1
T677T/A1298A	0/027	8	0/04	12	0/388

There is significant relationship between the control group and patients in heterozygous for both polymorphisms (035 / = 0P). But in other investigated cases in the table due to the P-value greater than 05/0, the assumption of a relationship between two variables is rejected. So there is no significant correlation between two variables.

RESULTS AND DISCUSSION

Infertile couples are a global problem, and according to the World Health Organization (WHO) report, approximately 80 million couples suffer from infertility. In about half of all cases of infertility, male factor involved. Given the high prevalence of infertility in men as well as high costs that would be spent annually on treatment, study and analysis of the factors affecting the disease in men is of particular importance. Given that male infertility is a heterogeneous disorder resulting from multiple genetic and environmental factors, in spite of the many studies on its mechanism, since the molecular events that trigger the development of this disorder are not fully known. In recent years several reasons for male infertility, including chromosomal abnormalities, small deletions in chromosome Y, trans Location, cystic fibrosis and other genetic factors are known (Bhasin *et al.,* 1994). Today, it is known that genetic polymorphisms or a variation of certain genes involved in spermatogenesis is an important factor in male infertility. Some of these genes can be noted in the MTHFR gene. This encoding gene is a key enzyme cycle of folic acid. The polymorphisms in genes affecting in folic acid cycle may impair the biosynthesis of folate. Folate deficiency and its hyper homocysteinemia presented as the cause of many diseases, including male infertility. C677T and A1298C are two common Polymorphisms in the gene that encodes the MTHFR enzyme and reduces the enzymatic activity and impaired folate cycle and subsequently increase homocysteine and hyper methylation DNA and proteins (Brattstrom *et al.,* 1998). Of the two mentioned polymorphisms, C677T play more fundamental role in reducing the activity of this enzyme, especially in the homozygous state up to 70 percent reduce enzyme activity. C677T, C677T polymorphism and also increase homocysteine levels in the blood more than A1298C. DNA methylation is important in spermatogenesis process. The number of spermatids and the condensed spermatozias when the DNA methylation inhibited during spermatogenesis is, reduced, which can cause infertility in men (Ferlin and Foresta, 2005).

Because the DNA synthesis and its methylation is the main sections of spermatogenesis process, thus folate metabolism also indirectly in the process will be important. So, polymorphisms of MTHFR gene involved in folate cycle can be associated with male infertility (Ebisch *et al.,* 2003). The relationship between this genes and disease the first time reported by Mudd and his colleagues (1972) about homocysteine disease and the then many disease caused by defects of this gene were found (Engbersen *et al.,* 1995). 5, 10- methylene tetrahydro folate reductase dysfunction causes homocysteine cannot be converted to methionine and accumulation in the blood and leads in hyper homocysteinemia. By increasing the amount of homocysteine in the blood, some enter in into the urine, leading tohomosystenoria, this disease show range of clinical signs. The T677T homozygous individuals are prone to hyper homocysteinemia. As MTHFR activity to produce 5-methyl tetra-dihydro folate and thus, reduce homocysteine, is low (Brown, 2001). In the remaining activity of the enzyme defects less than 20% due to increased homocysteine plasma and urinary disorders depending on the age of onset and severity of the disorderit create , a different phenotypic protests including developmental delay, mental retardation, mental health problems like schizophrenia and degenerative diseases system neurological, movement problems, muscle weakness and seizures (Dallaire and Huret, 2005). However, as a result of the enzyme deficiency, the balance of the conversion reaction of uracil into thymine, it will be created ,increase uracil and reduce the thymine and finally increase uracil conversion into the DNA, and May during the process of repair a withdraw of uracil, a failure of double-stranded DNA may be shaped. As a result of chromosomal abnormalities that is a factor for cancer is increasing. In certain cancers, MTHFR polymorphisms affecting through interaction with folate cycle on DNA methylation and by increased risk of some cancers are associated. This enzyme deficiency leads to deficiency in the DNA methylation. Hayprmthylation is effective by suppressing tumor suppressor genes and Haypvmtylasyvn DNA by activating oncogenes to create tumors.

According to studies, this gene polymorphism with other diseases such as depression and coronary artery disease are associated. But the role of this polymorphism in the creation of this disease is still controversial (Botto and Yang, 2000).

MTHFR deficiency by reducing the sources of methionine prevents a variety of methylation of substrates including proteins, RNA, DNA and his tones. The situation worsens by accumulation of S-Adenosyl homocysteine. If the process of trans-methylation could not follow with cell division, cell survival decreases. Trans-methylation reactions (methylation DNA) are essential for normal spermatogenesis, because methylation participate in the normal maturation of germ cells, gene expression and genomic events. High levels of expression of MTHFR in testis ensures that sufficient amounts of folate derivatives move toward the main producing methyl group -S adenosyl methionine that is the main donor of the methyl group in the methylation reaction. Thus, changes in the supply of methyl resulting from MTHFR deficiency can affect on spermatogenesis. In addition, inactivation of cellular nucleotide of MTHFR damage cell nucleotide which is effectively involved in cell proliferation, as rapid cell proliferation is necessary precursors of male germ cells, MTHFR, can play a significant role in this process (Cravo *et al.*, 1996).

In the study that was done on male mice lacking MTHFR, lack of spermatogenesis and finally, complete infertility was seen. The male rats adult of MTHFR - / -had unusual histology testicular with devoid tubules of germ cells and rarely mature germ cells were observed. This study confirmed that the defect in the MTHFR cause abnormal spermatogenesis and male infertility (Bailey and Gregory, 1999). In this research the relationship between C677T and A1298C polymorphisms of MTHFR gene in infertile men were studied.

Until now, the relationship between folic acid cycle deficiency and infertility among men in some populations has been studied. C677T polymorphism in Korean population has been associated with male infertility. In India's population relationship between C677T and A1298C polymorphisms and male infertility has been found. Also separately examination of polymorphisms of MTHFR gene with male infertility in various countries is available.

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