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Biochemical and molecular study in diabetic infertile males

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Abstract--As per the statement by World Health Organization, Infertility is presence of a medical condition of the couple with inability in getting conceived for 1 year or more without usage of any contraception. In the present situation, the incidence of Infertility has increased and it is viewed as a major concern with affecting about 15% to 20% of the couples. A detailed systematic examination of history, investigative protocol is necessary for proper management of infertility. The patients were also advised for blood hormonal analysis tests such as Follicle Stimulation Hormone (FSH), Testosterone, Luteinizing Hormone (LH), Prolactin. Methylene tetrahydrofolate reductase (MTHFR) and methionine synthase reductase (MTRR) are the key enzymes in folate metabolism. The enzyme activities of MTHFR and MTRR are influenced by gene polymorphisms. So the polymorphisms of MTHFR and MTRR may be a potential risk factor for male infertility. The present study was done in 100 males, 50 diabetic infertile and 50 non-diabetic fertile men. Semen samples were obtained and analyzed as per the instructions given in the WHO manual for semen analysis [10]. A blood sample was collected and analyzed for the levels of FBS, FSH, LH, Prolactin and Testosterone. The results were arranged in suitable tables for analysis under the relevant headings. The results were averaged as (mean \pm standard

deviation) for each parameter subgroups separately. Total genomic DNA was extracted from blood sample. The MTHFR C677T polymorphism was analyzed by polymerase chain reaction (PCR). The amplification protocol was also followed. The test results from the PCR for both polymorphisms were confirmed by RFLP/PCR using restriction enzymes *HinfI* and *MboII*. The digestion products were evaluated and then the genotype was determined. Each genotype determined by electrophoresis was evaluated by DNA sequencing for verification. Data were analyzed using the SPSS statistical package, version 20. The analysis confirms that there is a significant decrease in the normal Sperm morphology, count and motility of the infertile group when compared to normal non-diabetic fertile males. FBS, FSH, LH and Prolactin were found to be increased while the levels of Testosterone observed to be reduced. With regard to the frequencies of the 2 genotypes and alleles of MTHFR A1298C, there were no statistically significant differences between the patient group and the control group.

Keywords---Diabetic infertile, biochemical, semen analysis, FSH, LH, FBS, Prolactin, Testosterone, MTHFR.

Introduction

As per the statement by World Health Organization, Infertility is presence of a medical condition of the couple with inability in getting conceived for 1 year or more without usage of any contraception. In the present situation, the incidence of Infertility has increased and it is viewed as a major concern with affecting about 15% to 20% of the couples [1–4]. Significance of Infertility has increased as it may be associated with mental agony and psychological disturbances of an individual, further which may even lead to suicide. Because of these conditions, the couples who are coming to Infertility clinics or hospitals shall be taken care with proper counselling. When the prevalence of infertility cases are analyzed, it has been found that 40% to 50% of factors leading to infertility are related to only males [5–6].

A detailed systematic examination of history, investigative protocol (microscopic and macroscopic semen analysis, hormonal and molecular analysis) are necessary for proper management of infertility. WHO has published a manual describing the details of normal parameters of normal semen along with it also contains the procedures involved in proper analysis of semen in the laboratories [7]. The patients were also advised for blood hormonal analysis tests as these hormonal tests are more reliable and valid in the further proper management of the infertility [8]. These hormonal tests include Follicle Stimulation Hormone (FSH), Testosterone, Luteinizing Hormone (LH), Prolactin [9-17].

Folate plays an important role in cell metabolism, like the synthesis of nucleic acids and epigenetic regulation of gene expression through remethylation of homocysteine into methionine [18]. Once the folate is deficient, the proliferation of sperm cells will be reduced.[19] Methylene tetrahydrofolate reductase (MTHFR)

and methionine synthase reductase (MTRR) are the key enzymes in folate metabolism. The enzyme activities of MTHFR and MTRR are influenced by gene polymorphisms.[20] So the polymorphisms of MTHFR and MTRR may be a potential risk factor for male infertility.

Materials and Methods

The present cross section study was done in 100 males, 50 diabetic infertile men (50%) and 50 non-diabetic fertile men (50%). After obtaining Institutional Ethical Committee approval for the study, male patients attending Department of General Medicine, Dr.Patnam Mahender Reddy Institute of Medical Sciences, Hyderabad, Telangana during January 2020 to December 2021 have been selected as per the inclusion and exclusion criteria with complete medical and clinical histories. Diabetic infertile men and non-diabetic fertile men with age between 25 to 45 years are selected after obtaining an inform consent. Men with varicocele, smoker, taking exogenous testosterone or any other hormonal drugs, Anti-hypertensives, supplements or non-prescribed medications such as anabolic steroids, having retrograde ejaculation were excluded from this study.

A blood sample was withdrawn from a cubital vein of each participant, centrifuged, and the serum was separated and frozen. All samples were analyzed at Central Laboratory, Dr.Patnam Mahender Reddy Institute of Medical Sciences. Serum samples were measured for the levels of FBS, FSH, LH, Prolactin and Testosterone by Chemiluminescence Immunoassay (CLIA) using Beckmen Coulter analyzer Access-2 auto-analyzer [16-20]. Control materials were used in conjunction to patients' samples in every analytical runs.

The results were arranged in suitable tables for analysis under the relevant headings. The results were averaged as (mean \pm standard deviation) for each parameter subgroups separately. Each variable, including Semen Analysis, FBS, FSH, LH, Testosterone and Prolactin were analysed by Paired Sample t-test.

Total genomic DNA was extracted from blood sample by using Qiagen QIAcube Connect with QIAamp DNA mini QIAcube kit (QIAGEN India Pvt. Ltd.) as per the manufacturer's protocol. The MTHFR C677T polymorphism was analyzed by LightCycler[®] 96 polymerase chain reaction (PCR) (Roche Diagnostics India Pvt Ltd). Specific primer pairs were selected according to the related reference sequence (5'-TGA AGG AGA AGG TGT CTG CGG GA-3' as the upstream primer and 5'-AGG ACG GTG CGG TGA GAG TG-3' as the downstream primer for MTHFR C677T [21]; 5'-CTT TGG GGA GCT GAA GGA CTA CTA C-3' as the upstream primer and 5'-CAC TTT GTG ACC ATT CCG GTT TG-3' as the downstream primer for MTHFR A1298C [22].

PCR reactions were performed in a final volume of 10 μ l containing 1 LightCycler master mix hybridization probes from Roche Diagnostics, magnesium chloride (3.5 mmol/L), 677-primer mix (677F/677R, 0.5 μ mol/L of each), 1298-primer mix (1298F/1298R, 0.5 μ mol/L of each), 677-probe mix (677-HP1/677HP-2, 0.2 μ mol/L each), and 1298-probe mix (1298-HP1/1298-HP2, 0.2 μ mol/L each). Water (2 μ l; Roche) was added to make up the volume to 8 μ l per assay. Template DNA (2 μ l, concentration as eluted from the extraction columns) was pipetted into

LightCycler capillaries (Roche) containing 8 μ l of master mix. Each test run included a reagent blank, in which template DNA was replaced with PCR-grade water. Capillaries were capped, centrifuged briefly at 1000 g and placed into the LightCycler instrument. The amplification protocol was as follows: one cycle of 30 seconds at 95°C followed by 45 cycles consisting of denaturation at 95°C for 4 minutes, annealing at 52°C for 10 seconds, and extension at 72°C for 10 seconds at a transition rate of 20°C/second. The emitted fluorescence was measured at the end of the annealing phase in each cycle. All analyses were performed with color compensation using reagents from Roche Diagnostics. The test results from the LightCycler PCR for both polymorphisms were confirmed by RFLP/PCR using restriction enzymes *Hinf*I and *Mbo*II (both from New England BioLabs, Boston, MA) as described previously [21,22].

The digestion products were evaluated on an ethidium bromide-stained agarose gel (3% for MTHFR C677T, 4% for MTHFR A1298C) and then the genotype was determined. Each genotype determined by electrophoresis was evaluated by DNA sequencing for verification. Data were analyzed using the SPSS statistical package, version 20 (SPSS, Inc., Chicago, IL, USA). A chi-square test was used to compare genotype and allele frequencies, with a P value <0.05 considered to be statistically significant. Odds ratios and 95% confidence intervals were calculated.

Results

The mean sperm concentration in infertile males (12.56 \pm 1.60) was found to be low when compared to fertile control group (14.20 \pm 1.80) which is a significant finding. Among the analysis of the data, the values having mean t-test with p value of (<0.05) was considered to be significant. In the study, it is found that the sperm concentration is directly related to the fertility with high value of normal counts when compared to the infertile group with lower counts so that we can easily confirm that the sperm concentration based on the observational statistical analysis data in the study (Table-1 & 2).

Observation of the findings in diabetic infertile males showed that there was a significant decrease in Sperm count (30.48 \pm 5.43; normal: 38.46 \pm 7.05); Sperm motility (29.68 \pm 7.31; normal: 39.74 \pm 7.71); Sperm morphology (3.10 \pm 0.70; normal: 3.74 \pm 0.44). The analysis confirms that there is a significant decrease in the normal Sperm morphology, count and motility of the infertile group when compared to normal non-diabetic fertile males (Table. 1 & 2). The p values of the means t-test was of p<0.05.

Table 1: Paired Samples Test between Non-Diabetic Fertile men and Diabetic Infertile men

	Paired Differences					t	df	Sig. (2-tailed)
	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
				Lower	Upper			
FBS (Control – Study)	-65.120	49.859	7.051	-79.290	-50.950	-9.235	49	.000
FSH (Control – Study)	-10.320	7.138	1.010	-12.349	-8.291	-10.223	49	.000

LH (Control – Study)	-5.760	3.261	.461	-6.687	-4.833	-12.489	49	.000
Testosterone (Control – Study)	3.280	2.339	.331	2.615	3.945	9.916	49	.000
Prolactin (Control – Study)	-8.200	5.518	.780	-9.768	-6.632	-10.508	49	.000

The Biochemical parameters such as FBS, FSH, LH, Prolactin and Testosterone are the major contribution of this study. The results of FBS, FSH, LH, Prolactin and Testosterone levels in infertile and control (proven non-diabetic fertile men) groups are analysed (Table 1). It is found that the serum levels of FBS (155.56±49.58; control value: 90.44±8.34), FSH (20.42±5.39; control: 10.10±4.27), LH (10.72±2.48; control: 4.96±2.03) and Prolactin levels (17.98±4.96; control: 9.78±4.25) were significantly elevated in infertile males when compared with fertile males (control). Serum Testosterone levels were found to be significantly reduced in diabetic infertile males when compared with non-diabetic fertile males (control).

Table 2: Paired Samples Correlations between Non-Diabetic Fertile men and Diabetic Infertile men

		N	Correlation	Sig.
Pair 1	FBS (Control & Study)	50	.051	.724
Pair 2	FSH (Control & Study)	50	-.077	.595
Pair 3	LH (Control & Study)	50	-.035	.811
Pair 4	Testosterone (Control & Study)	50	-.025	.862
Pair 5	Prolactin (Control & Study)	50	.291	.040

Table 3: Paired Samples Statistics between Non-Diabetic Fertile men and Diabetic Infertile men

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	FBS (Control)	90.44	50	8.345	1.180
	FBS (Case)	155.56	50	49.584	7.012
Pair 2	FSH (Control)	10.10	50	4.273	.604
	FSH (Case)	20.42	50	5.399	.763
Pair 3	LH (Control)	4.96	50	2.030	.287
	LH (Case)	10.72	50	2.483	.351
Pair 4	Testosterone (Control)	4.98	50	2.152	.304
	Testosterone (Case)	1.70	50	.863	.122
Pair 5	Prolactin (Control)	9.78	50	4.254	.602
	Prolactin (Case)	17.98	50	4.967	.702

The genotype distributions of MTHFR C677T and MTHFR A1298C in all study subjects were tested according to the Hardy-Weinberg law of genetic equilibrium ($P > 0.05$). The association between the MTHFR C677T genetic polymorphism and infertility status is shown in Table 3. The frequency of the MTHFR 677T allele was higher in the patient group than in the control group. Compared with the MTHFR 677CC genotype, the MTHFR 677TT genotype was associated with a greater likelihood of infertility. With regard to the frequencies of the 2 genotypes and alleles of MTHFR A1298C, there were no statistically significant differences between the patient group and the control group (Table 4).

Table 4: Genotype and allele distribution of MTHFR SNPs in Non-Diabetic Fertile men and Diabetic Infertile men

SNP	Genotype	All Subjects (N=100)	Control (N=50)	Patients (N=50)	P value	OR (95%CI)
C677T	CC	50	26 (52%)	24 (48%)		
	CT	43	21 (42%)	22 (44%)	0.856	1.03 (0.70–1.51)
	TT	05	03 (6%)	02 (4%)	0.224	1.21 (0.69–2.13)
	C allele	143	73 (54%)	70 (70%)		
	T allele	53	27 (54%)	26 (26%)	0.366	1.08 (0.84–1.40)
A1298C	AA	49	27 (54%)	22 (44%)		
	AC	40	19 (38%)	21 (42%)	0.247	1.29 (0.88–1.9)
	CC	11	04 (8%)	07 (14%)	0.782	1.36 (0.45–4.16)
	A allele	138	73 (73%)	65 (65%)		
	C allele	62	27 (27%)	35 (35%)	0.181	1.26 (0.9–1.75)

Discussion

The present study has been done on 50 diabetic infertile men as study group and 50 diabetic fertile men as the control group. Increased concentrations of FSH and LH are also reported by other authors [23–27] in infertile males. Similarly the elevated levels of LH in persons with oligozoospermia and azoospermia were also reported [28,25]. Significant differences in the levels of testosterone between fertile and infertile men were also reported [26,27]. FSH, LH and Testosterone are required for production of spermatozoa. FSH acts directly on the seminiferous tubules whereas LH stimulates spermatogenesis indirectly via testosterone. FSH also plays a key role in stimulating mitotic and meiotic DNA synthesis in spermatogonia [29].

Folates, the group B vitamins are having significant role in the metabolism of cell such as nucleic acids synthesis and regulation of expression of through remethylation [29]. It has been observed in some studies that these enzymes involved in folate metabolism are having a significant role in spermatogenesis [26,30]. Improper folate leads to DNA hypomethylation which is responsible for inducing uracil mistakenly at the time of DNA synthesis which results in formation of errors in DNA repair, strand brittleness and anomalies of chromosomal [30]. It is also found that decreased levels of folate in seminal plasma is related to higher sperm DNA damage [31].

In the present study, the association of the two Single nucleotide polymorphisms (SNPs) (MTHFR C677T and A1298C) of one folate-related enzyme genes with male infertility are studied. Both MTHFR C677T and A1298C are found to linked with lowered enzyme activity [32,22]. This occurrence of the alleles showed variation among different studies among the ethnic population regarding the association of MTHFR gene polymorphism with male infertility [23-28, 33–44]. The association of polymorphism in MTHFR C677T was observed in persons with male infertility in the German [44], Indian [27,35,39,40], Korean [25], Chinese [45] and Italian men [42]. In certain other studies, there was no statistical significant correlation of C677T variation with male infertility at Italy and Netherlands [23, 43]. In our study, we did not find any association of C677T polymorphism with male infertility in the study groups. The frequency of the MTHFR A1298C variant allele also differs across populations, the frequency of the C allele is less frequent among some of the population [46]. No association between MTHFR A1298C and male infertility was observed in the studies conducted on French, Indian, Korean and Moroccan populations [39, 34, 38]. The study can be made even more relevant and significant by including the SNP studies of MTR A2756G and MTRR A66G. further it would be more relevant if cytological studies such as Y Chromosome microdeletion, FISH, Karyotyping also are included in the study.

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