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Research Article

Characteristics of *MTHFR* C677T and A1298C Polymorphisms in Infertile Men and Impact of 3 Weeks of Yoga on *MTHFR* gene Expression: A Clinical Case Report

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Abstract

Introduction: One of the enzymes of the folate metabolism is methylenetetrahydrofolate reductase (MTHFR). The *MTHFR* gene polymorphism is correlated with diverse disorders (cardiovascular, renal, congenital abnormalities, pregnancy outcome, cancers, diabetes, and psychiatric illness etc. including male infertility). DNA methylation being an important epigenetic feature plays a critical role in the course of spermatogenesis. Sperm DNA is prone for oxidative stress-induced damage which also disrupts global sperm DNA methylation and might play crucial role in male infertility causation. *MTHFR* C677T (rs1801133, pAla222Val) and A1298C (rs181131, pGlu-Ala) polymorphisms are widely investigated polymorphisms resulting in low enzymatic activity which is connected with male infertility. Nevertheless epidemiology of these two important polymorphisms differs based on geography and ethnicity. And non-uniform results have been obtained across various studies in different populations. The present study was aimed at examining the association of *MTHFR* polymorphism in infertile men in an Indian subpopulation and to study the impact of yoga on *MTHFR* gene expression.

Methods: We screened 30 infertile men and 30 healthy fertile men for polymorphic variants of two important polymorphism in *MTHFR* gene at 677 and 1298 position. Genomic DNA was isolated from blood by salting out method. We employed PCR-RFLP techniques using site specific primers for C677T and A1298C polymorphism followed by HinfI and MboII digestion respectively to assess the polymorphisms. We also enrolled the cases for undergoing Yoga Based Lifestyle Intervention (YBLI) for 3 weeks to evaluate the impact on seminal oxidative stress levels and changes in relative expression of *MTHFR* gene in the participants from baseline.

Results: To calculate the p-value for observed genotype and allele frequencies of *MTHFR* C677T and A1298C polymorphism Fisher's exact test was performed. Odds ratios, 95% confidence interval, relative gene expression were determined by suitable statistical tool. Our results revealed that CT genotype of *MTHFR* C677T polymorphism was significantly associated with increased risk of male infertility (OR=5.4321; 95% CI=2.2459-13.1569, p<0.0002). A combined odds ratio of CT and TT genotypes in cases against controls was calculated and found significant (OR=5.8689; 95% CI=2.4403-14.1180, p=0.0001). Likewise for *MTHFR* A1298C polymorphism AC genotype was found to have significant association (OR=13.2564; 95% CI=5.0787-34.6019, p<0.0001) with male infertility risk. Combined odds ratio of AC and CC genotypes in cases against controls also demonstrated to be significant (OR=16.4701; 95% CI=6.3855-42.4812, p<0.0001). We found significantly higher levels of seminal oxidative stress marker ROS (p-value <0.0118) in cases as compared to the controls. There was a significant decline in levels of seminal free radical levels post yoga intervention. The average relative change in *MTHFR* expressions was 5.517974 axis fold change with concomitant significant decrease in seminal ROS (p-value <0.05) in the participants post YBLI (day 21) as compared to baseline (day 0).

Conclusion: In summary this report revealed that *MTHFR* C677T and A1298C polymorphism combined with ROS mediated oxidative stress can act synergistically to impair the process of spermatogenesis which may cause male infertility. However YBLI, a simple life style intervention reduces the elevated free radical levels and thus may improve sperm DNA quality and also normalize transcripts irrespective of the genotype. Yoga may thus be used an adjunct therapy in management of male infertility.

Keywords: *MTHFR* gene; Male infertility; ROS; Oxidative stress; Yoga based intervention

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Introduction

Infertility is a complex yet common lifestyle disorder with emotional, psychological, economic and medical implications [1]. It affects 15-20% of couples in the reproductive age group [2]. Male factor is responsible in up to 40-50% of cases of infertility, of which in about 20% of the cases it is sole reason and in 30–40% it may be an ancillary factor [3]. Approximately in only half of the cases of male infertility a clear cause can be identified [4]. Those in whom no definite cause is found are labelled as 'male infertility of unknown origin' which is further sub-classified into idiopathic and unexplained infertility. In idiopathic and unexplained variety there can be occurrence of variable levels of DNA damage leading to qualitative decline in semen quality. Higher levels of seminal Oxidative Stress (OS) have been observed in

infertile men with normal semen parameters due to increased Reactive Oxygen Species (ROS) [5]. OS is a condition of ROS production in excess of the buffering capacity of body's antioxidants. Presence of a baseline level of seminal ROS is required for regulating normal physiological sperm functions. Because sperm loses majority of its antioxidants during its morphological maturation process, sperm is especially vulnerable to elevated ROS levels. High levels of ROS causes lipid peroxidation of sperm plasma membrane, damages axoneme, DNA, RNA, proteins and also impair mitochondrial functions [6]. Therefore elevated ROS level is detrimental and can adversely impact sperm membrane fluidity and permeability which by causing abnormalities in maturation, capacitation and functions of sperm might eventually affect the process of fertilization. Recent studies have shown that genetic abnormalities are commonly encountered in idiopathic as well as unexplained male infertility which have normal sperm parameters but with high seminal free radical levels and oxidative DNA damage.

MTHFR gene and male infertility

The process of spermatogenesis itself is a very complex phenomenon. Spermatogenesis is regulated by various genes involved in growth, differentiation, apoptosis and DNA damage and repair which in turn is associated with an important biochemical pathway, i.e. folate metabolism [7]. A number of Single Nucleotide Polymorphisms (SNPs) of genes involved in folate metabolism pathway have been held responsible for male infertility. The folate metabolism pathway involves a key enzyme methylenetetrahydrofolate reductase (MTHFR). In the interlinked folate-homocysteine-methionine pathway, substrate homocysteine is metabolized by one of the two pathways: remethylation or transsulfuration. In remethylation, homocysteine is transformed into methionine by transfer of a methyl group from 5-methyltetrahydrofolate. Methionine is further converted to S-Adenosyl Methionine (SAM), the universal methyl donor for methylation in the biological system. In the transsulfuration pathway the master antioxidant of the body, glutathione is generated from homocysteine. The MTHFR enzyme reduces 5, 10-methylene terhrdrofolate (THF) to 5-methyl THF. 5-methyl THF is again converted to 10-formyl THF which helps in the synthesis of purines and pyrimidines. It implies that MTHFR is an enzyme that by adding a methyl group to dietary folic acid make it usable, produces SAM, replenishes glutathione and contributes in nucleotide synthesis. Polymorphisms in the gene coding the MTHFR enzyme lead to lowering of MTHFR enzymatic activity, decrease intracellular folate pool and increases circulating homocysteine. Variant enzyme make the individual prone for various disorders including male infertility [8]. Till date a number of studies including meta-analysis have explored the associations between *MTHFR* gene SNPs and male infertility risk. The *MTHFR* locus is mapped to short arm of chromosome 1 at 1p36.22 and the gene is composed of 11 exons [9]. Different population studies till now have indicated that polymorphism of *MTHFR* C677T and A1298C is associated with decline in normal semen parameters and might have a role in male infertility causation. However, there are only limited data available from India with respect to *MTHFR* gene polymorphism and these studies remain conflicting rather than conclusive which may be due to inappropriate sample size, ambiguity in defining male infertility and related confounding factors including ethnicity.

Yoga and male infertility

Yoga a mind body intervention practiced in the Eastern societies and has blossomed throughout the globe gaining wide acceptance recently. The art and science of yoga comprises of a set of physical, mental, and spiritual practice exercises that initially emerged from the ancient India [10]. It was only in 19th century that yoga was introduced to western world. It is essentially a way of healthy living that focuses on bringing harmony between mind and body. The International Day of Yoga has been celebrated annually on 21stJune since 2015, after its inception in the United Nations General Assembly in 2014. Yoga practice blends the mind and body of an individual through coordinated breathing (pranayama), postural exercises (asanas), and meditation (dhyana). The practice of YBLI involves asana, meditation, pranayama, coordinated breathing exercises which have proven health benefits including rejuvenation and increased life expectancy. The four believable transduction pathways for these interventions are humoral factors, nervous system activity, cell trafficking and bioelectromagnetism [11]. Optimization of homeostatic set points, quenching of intracellular signalling noise, improved balance between homeostatic and non-homeostatic feedback loops are different postulated mechanisms which may act through these interventions [11]. Yoga and meditation practices lessen free radical levels and up-regulate antioxidant capacity. It may therefore improve DNA integrity [12]. Yoga Based Lifestyle Interventions (YBLI) reduces rate of telomere attrition and emphatically reinforce telomerase activity and cellular aging [12]. Yoga increases mitochondrial integrity and increases melatonin [13]. Melatonin is having oncostatic and strong antioxidant properties. A balance between oxidative stress and antioxidant components are of utmost importance for healthy living. If this balance is jeopardized, it can lead to several disorders including inflammation, cancer and aging [14]. Oxidative stress is implicated directly or indirectly in several pathological conditions in human body including male infertility and cancer. Regular YBLI increases antioxidant components and reduces oxidative stress biomarkers. It also enhances plasma serotonin levels and reduces plasma adrenaline levels to further benefit psychological stress [15]. Regular yoga augments antioxidant components and reverses oxidative stress, telomerase activity and oxidative DNA damage [12]. It also stabilizes baseline physiological functions through mechanism involving both afferent and efferent vagal activity. Its beneficial effect is seen in cardiovascular diseases, cancers, arthropathies, ocular disorders and immune dysfunctions as well as psychological disorders, such as anxiety or depression [16]. Overall effect of regular YBLI is that it enhances the antioxidant defence status. So oxidative stress is reduced which may protect from ROS mediated conditions including male infertility [17]. The current study was designed with the aim to understand the association of *MTHFR*C677T & A1298C polymorphisms and male infertility in Indian men. This study also evaluated the impact of 3 weeks of YBLI in *MTHFR* expression.

Material and Methods

Participants

The study included a prospective case control component with infertile male patients as cases and healthy fertile males as controls. A single arm prospective clinical trial was conducted in parallel to determine the pre and post impact of 3 weeks of YBLI on expression levels of *MTHFR* gene. A total of 60 participants were recruited after obtaining ethical clearance and informed written consent. There were 30 cases of infertility of unknown origin and 30 fertile controls who have fathered at least 1 child in last 2 years. All study participants were of Indo-Aryan ethnic origin and recruited from

the Andrology Clinic, AIIMS, New Delhi and were referred to our lab. For the prospective clinical trial those participants were enrolled who were able to complete 3 weeks of supervised YBLI with no recent change in lifestyle. Participants having obstructive pathology, other co morbidities like hypertension, diabetes, chronic respiratory illness etc., not agreed to undergo YBLI and those with recent lifestyle changes are excluded (Figure 1).

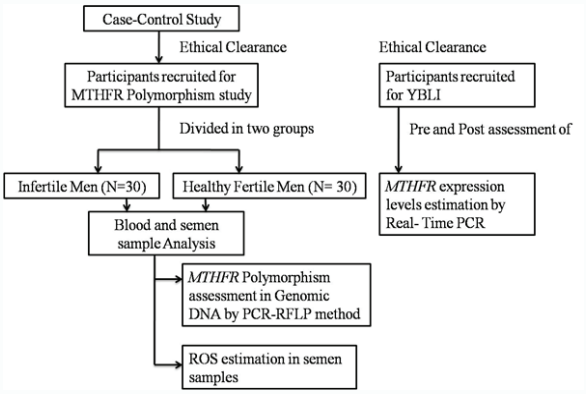

Figure 1: Schematic representation of the study design.

DNA isolation from peripheral blood

Peripheral blood samples (5 ml) were collected in kEDTA vacutainer tube. Genomic DNA was isolated using standard salting out method. The quality and quantity of isolated DNA was checked using Nanodrop and stored at –20°C until further use.

Semen Analysis

Semen samples were collected in sterile container. After liquefaction of the sample at 37°C, conventional semen analysis was performed (concentration, motility, morphology; as per WHO, 2010 guidelines).

Measurement of Reactive Oxygen Species (ROS) in semen

Seminal ROS levels were measured by the chemiluminesce method using luminal (5-amino-2,3-dihydro- 1,4-phthalazinedione, Sigma Chemical Co., St. Louis, MO, USA) in DMSO (di methyl sulphoxide) with Berthold luminometer (Berthold Detection Systems GmbH, Pforzheim, Germany). ROS levels were expressed as RLU/sec/million spermatozoa.

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism(PCR-RFLP) for Genotyping *MTHFR*

PCR-RFLP technique was employed to genotype *MTHFR* SNPs C677T (rs1801133) and A1298C (rs1801131) in genomic DNA of infertile males as well as healthy fertile male controls. Previously known forward and reverse primers sets and the PCR conditions used are described in Table 1. 15 µL of *MTHFR* 677 PCR amplicon of 477 base pair (bp) was synthesised using the known primers. The amplicon was then subjected to 1U of Hinf1 (Thermo Fisher Scientific) Restriction Enzyme (RE) digestion at 37°C for 15 hours as per the manufacturer's protocol and resolved in 3.5% agarose gel. The 'C' allele is cut by the enzyme and gives only two 425 and 52 bp products, while presence of 'T' allele establishes a site for Hinf1 digestion leading to digestion products of 260, 165, and 52 base pairs (bp) (Figure 2). 15µL of *MTHFR* 1298 PCR amplicon obtained was RE digested with 1 U of MboII (Thermo Fisher Scientific) at 37°C for 15 hours according to the manufacturer's specification and resolved in 3.5% agarose gel. The wild type allele 'A' yields a 56, 31, 30, 28, and 18 bp fragments; while the 'C' allele by abolishing site for MboII digestion yielded RE digestion products of 84, 31, 30, and 18 bp (Figure 3).

Yoga-based lifestyle intervention

A total of 30 infertile men were enrolled from October 2018 to September 2019 for the clinical trial involving YBLI. Detailed description of the pre-structured YBLI programme was provided to all participants. Supervised daily sessions were conducted for an average duration of two hours each day as guided by a certified yoga instructor. This integrated YBLI strategy comprises of a series of events as depicted in Table 2. Moreover each typical session was supplemented by interactive motivating session with the session instructor.

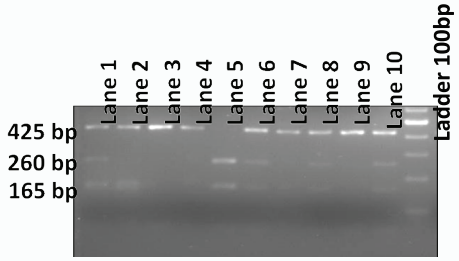

Figure 2: *MTHFR* C677T polymorphism was detected by PCR-RFLP. The Hinf1 restriction enzyme cleaved the 477 base pair (bp) PCR product. Whenever the 'C'allele is cut by the enzyme it yields 425 and 52 bp products, whereas cleavage at the 'T' allele yields 260, 165, and 52 bp products. Lane 1, 5, 6, 8. 10: CT heterozygous; Lane 3, 4, 7, 9: CC homozygous; Lanes 2 : TT homozygous; Lane 11: 100 bp ladder

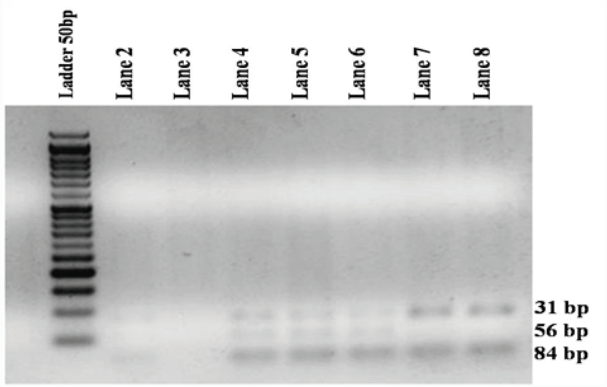

Figure 3: *MTHFR* A1298C polymorphism was detected by PCR-RFLP technique. The MboII restriction enzyme cleaved the 163 base pair (bp). Whenever the'A' allele is cut by the enzyme it yields 56, 31, 30, 28, and 18 bp, whereas cleavage at the 'C' allele yields 84, 31, 30, and 18 bp products. Lane 1: 50-bp ladder; Lanes 2, 3: AA homozygous; Lane 4, 5, 6: AC heterozygous and Lane 7, 8: CC homozygous.

Real time PCR (RT-PCR) protocol

Total RNA from blood is extracted by phenol-chloroform method. 1 ml of blood was mixed with RBC lysis buffer in DEPC water and mixed well. This step was repeated 2-3 times till a white cell pellet was obtained. Triazol was added, mixed thoroughly and centrifuged. Chloroform was mixed with the supernatant and centrifuged. After phase separation the middle phase was withdrawn and mixed with isopropanol and kept for 30 minutes. Mixture was centrifuged again and the supernatant discarded. The pellate was washed with 70% ethanol and recentrifuged and supernatant discarded. Obtained

Table 1: Primers used for C677T & A1298C polymorphisms. FP- forward primer, RP-reverse primer.

Name of SNP	Primer sequence (5' to 3')	PCR Cycle condition	Product size (bp)
MTHFR	FP: GGCTGTGCTGTGCTGTTG	94°C: 60s, 68°C: 60s,	
677 C > T	RP: CGCTCTGCAAGTTCTGGA	72°C: 60s	477
		(30 cycles)	
MTHFR	FP: CTTTGGGGAGCTGAAGGACTACTAC	94°C: 30s, 65°C: 35s,	
1298 A>C	RP: CACTTTGTGACCATTCCGGTTTG	72°C: 35s	163
		(30 cycles)	

Table 2: Details of yoga based lifestyle intervention (YBLI) programme.

Starting Prayer		**2 minute**
1. Yogic sukshmavyama	Finger loosening, Wrist loosening, Elbow loosening, Shoulder loosening, Toe bending, Ankle bending, Knee cap tightening, patella movement, Knee bending & Hip rotation	10 minute
2. Yogasana	Standing	5 minute
	Trikonasana	
	Katichakrasana	
	Tadasana	
	Veerabhadrasana	
	Sitting	5 minute
	Gomukhasana	
	Paschim-utaanasana	
	Shashaankasana	
	Vakrasana	
	Prone	5 minute
	Ek-pada-shalabhasana	
	Bhujangasana	
	Poorna- shalabhasana	
	Supine	5 minute
	Uttanapadasana	
	Setubhandhasana	
	Matsyasana	
3. Relaxation		10 minute
4. Pranayama	Kapalbhati (Kriya)	20 minute
	Ujjayi	
5. Nada anusandhana, A U M - A u m k a r recitation		3 minute
6. Dhyana (meditation)		10 minute
7. Shanti mantra-Closing prayer		2 minute
8. Relaxation		

RNA sample was air dried and DEPC water was then added. After quantification, the RNA sample was stored at -80°C till further processing. Reverse transcription of a total 1000 ng of RNA was done into complementary DNA (cDNA) using Agilent kit (AccuScript High Fidelity 1st Strand cDNA Synthesis Kit). Brilliant III Ultra-Fast SYBR Green quantitative polymerase chain reaction Master Mix was used for quantitative analysis of *MTHFR* gene using CFX96 real-time system (Bio-Rad, California, USA). The relative quantification of *MTHFR* was determined with 2−ΔΔCt method after normalizing with two internal housekeeping genes *β-ACTIN* and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*).

Statistical analysis

Descriptive statistics such as mean, Standard Deviation (SD), minimum and maximum were computed for all study parameters separately for each group (cases and controls). Difference in the study parameters between groups were compared using independent student 't' test. Hardy-Weinberg equilibrium for observed genotype frequencies for both the cases and healthy controls were tested using Chi square test. Odds ratio (OR) and 95% confidence interval (95% CI) for various alleles and genotypes were calculated. Statistical analysis was carried out with IBM software- Statistical Package for Social Sciences (SPSS) version 16 (SPSS Inc., USA). P value <0.05 was taken as statistically significant.Relative gene expression of *MTHFR* was expressed as fold change by 2-ΔΔCt method of the gene at baseline (Day 0) and at the end of active intervention (Day 21). Significance was considered as p value <0.05.

Results

We found significantly higher levels of ROS (p-value <0.0118) in cases as compared to the controls (Table 3).

We examined polymorphism in two SNPs of *MTHFR* gene, viz., C677T and A1298C using PCR-RFLP technique in genomic DNA of cases and controls. For *MTHFR* C677T, the occurrence of wild-type genotype CC was found to be lower in cases (46%) as compared to the controls (83.33%). The occurrence of the heterozygous genotype CT was found to be higher in cases (50%) as compared to the controls (16.66%) (p<0.0002) (OR=5.4321; 95% CI= 2.2459-13.1569). Homozygous genotype TT was found in 2 cases (4%) (p=0.0001) (OR=5.8689; 95% CI=2.4403-14.1180) and TT genotype was not found in controls. Similarly for *MTHFR* A1298C, the occurrence of wild-type AA genotype was lower in cases (18%) as compared to the controls (78.33%). The occurrence of heterozygous genotype AC was found to be higher in cases (66%) as compared to the controls (21.66%) (p<0.0001) (OR=13.2564; 95% CI= 5.0787-34.6019). Homozygous genotype CC were encountered in 8 cases (16%) (p<0.0001) (OR=16.4701; 95% CI= 6.3855-42.4812) compared to none found in any of the controls (Table 4).The images obtained from agarose gel electrophoresis for *MTHFR* C677T and A1298C are shown in Figure 2 and Figure 3 respectively. One-way analysis of variance (ANOVA) was conducted among cases for comparing ROS with combined genotypes of *MTHFR* 677 and 1298. ROS for heterozygous genotype AC/CT, AA/CT and AC/CC genotype was significantly higher compared with wild genotype AA/CC (all p<0.001).

MTHFR gene expression analysis by RT-PCR

Relative expression *MTHFR* gene in the participants were changed (upregulated) after 21 days of YBLI in comparison to day-0 i.e. prior to starting YBLI in the same group. The average relative change in gene expressions was 5.517974 axis fold change (Table 5). We also observed significantly lower levels of ROS (p-value <0.05) in the participants post intervention as compared to baseline ROS before starting the yoga programme (Table 6).

Discussion

MTHFR is one of the key enzymes of folate metabolic pathway which is crucial for DNA methylation and eventually spermatogenesis. Presence of polymorphic variant of *MTHFR* decreases MTHFR activity can subsequently affect DNA methylation. *MTHFR C*677T encodes for an enzyme variant which is thermolabile and upto 30-70% low in enzymatic activity [19] and thus, dysregulates methylation during spermatogenesis. *MTHFR* A1298C polymorphism also decreases activity of the enzyme up to 30% [20]. *Bezold et. al.* [21] in 2001 first highlighted the association of *MTHFR* C677T polymorphism and increased susceptibility of male factor infertility. It was followed by a bunch of molecular epidemiological studies consistently investigating the association but the findings were at odds with. Subsequent meta-

Table 3: Comparison of seminal oxidative stress biomarker ROS between cases and controls.

	Cases (n=31)	Controls (n=60)	Cut-off value (18)	p value
ROS values (RLU/sec/million)	29.37 ± 4.88 RLU/sec/million	26.31+5.62 RLU/sec/million	27.25 RLU/sec/million	0.0118

Table 4: Distribution of *MTHFR* A1298C and C677T alleles and genotypes in the peripheral blood DNA of cases and controls.

MTHFR Polymorphism	Cases (n=50)	Controls (n=60)	Odds ratio	95% CI	p-value
MTHFR C677T					
C Allele	72(72%)	110(91.66%)	1	-	
T Allele	28(28%)	10(8.33%)	4.2778	1.9594-9.3393	0.0003
CC Genotype	23(46%)	50(83.33%)	1	-	
CT Genotype	25(50%)	10(16.66%)	5.4321	2.2459-13.1569	0.0002
TT Genotypes	2(04%)	0	5.8696	2.4403-14.1180	0.0001
MTHFR A1298C					
A Allele	52(51%)	106(89.16%)	1	-	
C Allele	48(49%)	14(10.83%)	6.989	3.5352-13.8173	<0.0001
AA Genotype	9(18%)	47(78.33%)	1	-	
AC Genotype	33(66%)	13(21.66%)	13.2564	5.0787-34.6019	<0.0001
CC Genotype	8(16%)	0	16.4701	6.3855-42.4812	<0.0001

Table 5: Relative expression of *MTHFR* with respect to β-*ACTIN* and *GAPDH* (average ΔCt) in blood samples of infertile men and AFC in the gene expression post-yoga with respect to pre-yoga. Values are expressed as mean (n=30). GOI, genes of interest; AFC, axis fold change.

GOI	ΔCt Pre-yoga	ΔCt Post-yoga	$2^{-\Delta\Delta Ct}$	AFC
MTHFR	1.571209	0.617115	-0.95409	**5.517974**

Table 6: Comparison of ROS in participants at baseline and at the end of YBLI intervention.

Experimental Parameter	Baseline (Day 0)	End of intervention (Day 21)	P-value
ROS	37.21 (5.56 to 163.14)	22.36 (0.89 to 116.36)	<0.05

analyses were conducted to obtain precise information by minimising the limitations of individual studies. *Wu and Y. Yang et al.* found that the *MTHFR* C677T polymorphism is responsible for risk of infertility in Asian males, but not in Caucasians. *Shen et al.* also observed association of *MTHFR*A1298C polymorphism and male infertility risk in their meta-analysis. *Y Yang et al.* however observed significant association of *MTHFR* A1298C with male infertility susceptibility in all population groups. Recent study by *Gong et al.*, *Hong et al.* observed strong association between *MTHFR* C677T polymorphism and the risk of male infertility [19]. *Zhang et al.* in 2017 in their meta-analysis observed that *MTHFR* A1298C polymorphism may be a potential risk factor for male infertility, particularly in Asian population [22]. *Tian-Lu Shi et al.* in 2018 in their meta-analysis highlighted that *MTHFR* C677T andA1298C polymorphisms increase susceptibility to male infertility in Asians [23]. In contrast *Camprubi C et al., Ravel C et al., Wuhua Ni et al.,* could not find enough evidence to correlate *MTHFR* C677T polymorphism with risk of male infertility. *Eloualid A et al.* indicated a lack of association of the *MTHFR* C677T polymorphism with male infertility in *Moroccan* population, nevertheless they reported homozygous (CC) genotype of *MTHFR* A1298C at a statistically high significance in severe oligozoospermia cases [24]. Contrasting findings were encountered in studies from Indian subcontinent [25-30]. Our study in concordance with most of the published studies indicated that *MTHFR* C677T and A1298C polymorphisms are directly linked with genetic susceptibility of male infertility. Heterozygous CT genotype of *MTHFR* C677T variant demonstrated strong association with asthenozoospermia (p<0.0001) and normospermia (p=0.0083) groups in sub-group analysis of the infertile cases. Also the frequency of 'T' allele was significantly higher in each infertile sub-group (all p<0.05). Likewise the 'C' allele and heterozygous AC genotype of *MTHFR* A1298C variant revealed strong association in all the infertile subgroups (all p<0.05) [Data not shown]. Overall, the occurrence of genotypes CT+TT and AC+CC of both the polymorphism was significantly more in infertile cases compared to healthy controls (all p <0.05). Unhealthy lifestyle habits such as tobacco chewing, smoking, alcohol consumption, exposure to toxic chemicals, sedentary and highly stressed lifestyle, physical inactivity and various environmental factors influence the sperm DNA quality which eventually can determine the reproductive outcomes [31]. Owing to limited antioxidant defence capability and inefficient mechanisms for detection and repair of DNA damage, the mature spermatozoa are at more risk for DNA damages [32]. In our study, we have found statistically significant higher levels of oxidative stress marker ROS in infertile cases (29.37±4.88 RLU/sec/million) as compared to the fertile controls (26.31+5.62 RLU/sec/million). This finding further emphasized that higher oxidative stress in infertile cases increases oxidative DNA damage in sperms which might be playing significant role in causation of male infertility. We found elevated ROS levels in the heterozygous CT genotype (for *MTHFR* 677) and AC genotype (for *MTHFR* 1298) as compared with the wild-type genotypes for both the SNPs. We also noticed that ROS value was significantly higher in combined heterozygous genotype AC/CT, AA/CT and AC/CC compared with wild genotype AA/CC (all p<0.001). This observation can be explained as higher oxidative stress is expected in individuals with polymorphic variant with less MTHFR enzyme activity in whom replenishment of glutathione is not as fast as it would be in a case with a normal genotype. A significant association (all p<0.05) between ROS and *MTHFR* combined polymorphic genotypes were also noted for the cases but not for the healthy fertile controls. We observed that the frequency of heterozygous genotype CT (for *MTHFR* C677T) and genotype AC (for *MTHFR* A1298C) was higher in cases. The genotype CT for *MTHFR* SNP 677 and genotype AC for *MTHFR* SNP 1298 are at higher risk (OR being 5.43 for CT genotype and 13.26 for AC genotype respectively) for male infertility. The results also indicated a significantly (p <0.05) higher 'T' allele in C677T and 'C' allele in A1298C variants. Furthermore heterozygous genotypes of both the polymorphism were more in almost all semen groups of infertile men as compared to the controls. The present study suggested the possible association of *MTHFR* C677T and A1298C polymorphisms and male infertility occurrence. The underlying mechanism may be due to global non-availability of SAM required for methylation reactions because of decrease activity

of MTHFR enzyme which may also result in global hypomethylation of the genomic DNA. Hypomethylation may lead to deletions of important regulatory regions in the genome which unmask repetitive elements and transposons and accordingly can affect transcription and replication [33]. Our findings re-emphasized the finding of most of the previous studies highlighting the important role of *MTHFR* gene polymorphism with an increased risk of male infertility in Indian subpopulation. Our findings also reiterate that oxidative stress, *MTHFR* polymorphisms and resultant DNA damage altogether can act synergistically in the impairment of spermatogenesis thereby leading to increase in the risk of male infertility.

Study of Yoga Based Lifestyle Intervention on *MTHFR* gene expression

Yoga based interventions have shown tremendous benefits in many medical conditions arising out of stress, lifestyle related disorders, chronic inflammatory conditions like rheumatoid arthritis and in metabolic syndromes [12,34,35]. YBLI increases sperm quality by decreasing oxidative stress, inflammation and various cellular stress markers [36]. We have evaluated the impact of YBLI on *MTHFR* gene expression levels. This trial comprising 3 weeks of yoga based intervention revealed that post intervention (21 days) versus baseline (day 0), there is more than fivefold increase in relative *MTHFR* gene expression with concomitant significant decrease in the oxidative stress as shown by significant decrease in seminal ROS. These findings can be linked to increased *MTHFR* expression leading to MTHFR enzyme becoming more capable of mitigating oxidative stress-induced aberrant sperm DNA methylation which would have affected the sperm epigenome more. The findings underscore that regular practice of yoga although for a brief duration of 3 weeks could upregulate expression levels of *MTHFR*. This might be more important in sperm considering its more vulnerability to oxidative stress and also because MTHFR enzyme activity is highest in testes. Therefore yoga based lifestyle can be useful as an adjunct therapy in management of male factor infertility especially idiopathic &unexplained infertility. Earlier research from our lab have consistently revealed and documented that yoga upregulates expression of anti-inflammatory genes, upregulates expression levels of various BER pathway genes and decreases expression of pro-inflammatory genes [12,18,35,37]. Yoga is also found to improve mitochondrial function leading to production of more ATP and fewer free radicals [38]. Another recent study revealed improvement in mitochondrial integrity and increment of fertility potential after adopting yoga based lifestyle in rheumatoid arthritis patients [39]. The highlight of this study is that it is the first report of increased expression of *MTHFR* gene and decline in free radical levels following practice of yoga. What is also noteworthy in this study is that this simple, economic lifestyle intervention can be adopted as an adjunct to the standard treatment options in a resource limited set up considering the burden of infertility and cost of treatment.

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