Association of NRF2 Gene Polymorphism (RS6721961) with Semen Parameters of Infertile Males in Pakistani Population

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ABSTRACT

Objective: To determine an association of single nucleotide polymorphism of the NRF2 gene (RS6721961) with semen parameters of primary male infertility patients in the Pakistani population.

Study Design: Comparative cross-sectional study.

Place and Duration of Study: Department of Biochemistry, Islamic International Medical College, Rawalpindi, in collaboration with two private infertility clinics (American Infertility Center, Rawalpindi and Mother and Child International Hospital and Research Institute, Mirpur, AJK) from Oct 2020 to Sep 2021.

Methodology: A total of 288 participants were included in this study. There were 144 diagnosed cases of primary male infertility and 144 healthy fertile males, age and ethnicity matched controls. Blood samples were collected from participants after obtaining written informed consent. DNA was extracted by the Chelex TM Methodoogy. Multiplex PCR was done to determine the respective allelic frequencies of NRF2 (RS6721961) genotypes using specific primers.

Results: There was no significant association between NRF2 genotypes and semen volume and semn colour in infertile males (p-value 0.32 and 0.84, respectively). Out of 144 cases, 111 (77%) had normal sperm count, 29 (20.1%) had oligospermia, while 4 (2.7%) patients had azoospermia. A significant association was observed between NRF2 genotypes and sperm concentration of infertile males (*p*-value <0.001). Out of 144 cases, 80 (55.5%) had normal motility, 57 (39.5%) had asthenospermia while 7 (8.4%) patients had necrospermia. A strong association was observed between NRF2 genotypes and sperm motility in infertile males (*p*-value <0.001).

Conclusion: Single nucleotide polymorphism (RS6721961) of the NRF2 gene is significantly associated with sperm concentration and sperm motility in infertile males in the studied Pakistani population.

Keywords: Male infertility, Nuclear factor erythroid 2-related factor 2 (NRF2), Polymerase chain reaction, Single nucleotide polymorphism.

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INTRODUCTION

Infertility affects approximately 70 million couples worldwide, with male factors accounting for over half of the cases.[4] In Pakistan, the pooled prevalence of primary male infertility was 76.19%, reported in 2015.[1,2]

Gene polymorphism refers to multiple forms of a single gene.[3] Single nucleotide polymorphism in a genome is a single nucleotide change that aids in determining the link between gene variations and prevalent disorders.[4,5] Male infertility mainly accounts for sperm abnormalities, and many of them are caused by genetic defects. Genetic factors are found in about 15% of infertile males, including chromosomal abnormalities or single-gene mutations.[6] The nuclear transcriptional protein, erythroid two related factors 2 (NRF2), induces antioxidant enzymes via the signalling pathway of the antioxidant response element (ARE).[7] NRF2 protects the male reproductive tract from oxidative stress by playing a vital role in cellular antioxidant defence during spermatogenesis and fertilisation.[8] Numerous genes are involved in normal spermatogenesis and sperm functions. Oxidative stress (OS) is a significant contributor to a decline in male fertility by producing irreparable damage to DNA, lipids, proteins and enzymatic systems, ultimately leading to cell death and a reduction in semen parameters linked to male infertility.[9] Many studies have reported that impairment of the NRF2-ARE signalling pathway and functional polymorphisms of the NRF2 promoter region is associated with many human diseases, including male infertility.[10]

There is limited information and statistical data available regarding the genetic basis of infertility in Pakistani males. Hence, it is important to carry out genetic studies for male infertility to decrease the

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hassle of invasive infertility testing of related females and further irrelevant tests of the concerned males to reduce the financial burdens and the associated anxiety. Therefore, the current study was carried out to find the allelic frequencies and an association of NRF2 gene polymorphism (RS6721961) with semen parameters of infertile males in the Pakistani population.

METHODOLOGY

This comparative cross sectional study was conducted from October 2020 to September 2021 at the Department of Biochemistry, Islamic International Medical College, Rawalpindi, in collaboration with two private infertility clinics (American Infertility Center, Rawalpindi and Mother and Child International Hospital and Research Institute, Mirpur, AJK). Permission from the Ethics Review Committee via letter number Riphah/IIMC/IRC/20/151 was sought prior to the commencement of this study.

The sample size was calculated using the formula $n = \frac{z^2 qp}{d^2}$ where level of confidence was taken as 1.96, disease prevalence was 76.19%, and the margin of error was chosen at 5%.[11] The sample size of 288 was calculated and non-probability convenience sampling was carried out.

Inclusion Criteria: Diagnosed patients of primary male infertility between 25-55 years of age belonging to any ethnicity were included in this study. This study also included healthy fertile males, age and ethnicity matched controls.

Exclusion Criteria: Male patients with secondary infertility, different metabolic and endocrine disorders including hypertension, diabetes mellitus, dyslipidemias, obesity, thyroid disorders, liver and kidney diseases, and those unwilling to participate were excluded from this study.

In the current study, already diagnosed cases of primary male infertility were included, and semen analysis reports were collected from them. Primary male infertility cases were subdivided based on impairment of semen parameters: 1) normospermia refered to cases with average sperm count (>15 million/ml), 2) azoospermia was defined as cases with absence of sperms in the ejaculate, 3) oligospermia refers to cases with sperm count <15 million/ml, 4) asthenospermia refered to cases with sperm motility <40% after 01 hours, 5) oligoasthenospermia refers to cases with sperm count <15 million/ml and motility <40% after 1 hour and 6) necrospermia refered to cases with dead sperms in ejaculate.[12]

After obtaining written informed consent, blood samples were collected from study subjects and stored at 4-8 C. DNA was extracted by the ChelexTM method and stored at-80 C in Eppendorf tubes until further analysis. NRF2 (RS6721961) gene was genotyped by Multiplex PCR using forward and reverse primers specific for amplifying the respective gene. Primers' sequences were designed using an article as a reference and were shown in the Table-I.

Table-I: Primers' sequences.

Primers	Primer Sequence, 5′ to 3′
Forward Primer (F1)	Ctccgtttgcctttgacgac
Reverse Primer (R1)	Ggggagatgtggacagcg
Forward Primer (F2)	Gcgaacacgagctgccgga
Reverse Primer (R2)	Ccctgatttggagttgcagaac

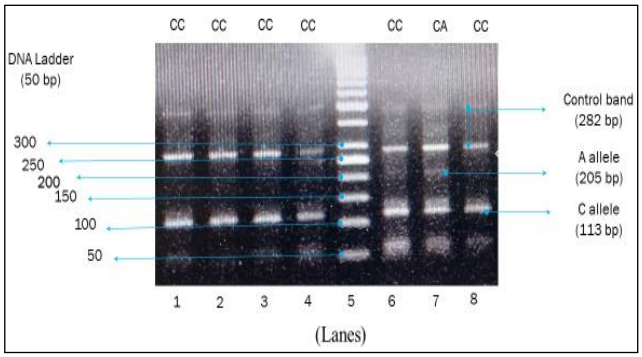

Figure: Amplified PCR products of NRF2 genotypes (RS6721961) separated on agarose gel electrophoresis. Lane 5 shows DNA reference ladder of 50 base pair. Lanes 1, 2, 3, 4, 6 & 8 show CC genotype in cases. Lane 7 shows CA genotype in cases.

Multiplex PCR technique was carried out in separate PCR tubes containing forward and reverse primers specific for the NRF2 gene. For each PCR reaction, the final total volume was 25 µl comprising of 8.5µl water by Invitrogen™, 12.5 µl 2x Thermoscientific™ Master Mix containing 0.05 U/µl Taq DNA polymerase, dNTPs & reaction buffer, 1 µl of primer mixture from 4 designed primers and 3 µl of extracted DNA sample to be genotyped.

PCR reaction procedure began with initial denaturation of DNA at 96°C for 5 minutes, followed by 35 amplification cycles, each comprising of denaturation at 96°C for 1 minute, annealing at 63°C for 1 minute, extension at 72°C for 1 minute and the final extension step for the gene of interest was done at 72°C for 5 minutes. After completing 35 cycles, the amplification was finished to hold at 4 °C. The reaction products were then subjected to Agarose gel electro-phoresis,

where 2g Agarose powder was mixed with 0.5 µg/ml of Ethidium Bromide in 1x TBE buffer. Gel electrophoresis was started with settings of current at 700mA and voltage at 100v for 45 minutes. Magnified DNA fragments were then visualized under a UV camera in Gene BoxTM by Gene System TM. Gene Ruler TM 50 bp by Thermoscientific TM was used as a DNA ladder to determine the sizes of magnified bands, as shown in Figure.

The statistical analysis was executed via Statistical Package for Social Sciences (SPSS) version 21. The chi-square test was carried out to determine the possible association between NRF2 SNP (RS6721961) genotypes and semen parameters of primary male infertility patients. Genotype frequencies and percen-tages were determined for descriptive statistics. The statistically significant difference was indicated by the *p*-value of ≤0.05.

RESULTS

This study included two hundred eighty-eight subjects, with 144 cases and 144 age and ethnicity matched-controls. Out of 144 cases, semen volume was normal in 137 patients (95.1%) and low in 7 (4.8%) cases. Semen color was normal in 141 patients (97.9%), pale yellow in 2 (1.3%) cases and hemorrhagic in 1 (0.6%) patient. No significant association of NRF2 genotypes was found with semen volume and semen colour of infertile males, with the *p*-value of 0.32 and 0.84, respectively, as shown in the Table-II.

Table-II: Association of NRF2 (RS6721961) gene polymorphism with semen volume and color in primary male infertility cases.

Genotypes	Semen Volume		*p*-value
	Normal 137 (95.1%)	Low 7 (4.8%)	
CC	94 (68.6%)	6 (85.7%)	0.32
CA	32 (23.3%)	-	
AA	11 (8.0%)	1 (14.2%)	
Genotypes	**Semen Color**		***p*-value**
	Normal 141 (97.9%)	**Pale Yellow 2 (1.3%)**	**Hemo-rrhagic 1 (0.6%)**
CC	98 (69.5%)	1 (50%)	1 (100%)
CA	31 (21.9%)	1 (50%)	-
AA	12 (8.5%)	-	-

Regarding sperm concentration, 111 patients (77%) had normal count, 29 (20.1%) patients had oligospermia while 4 (2.7%) patients had azoospermia. Significant association was observed between NRF2 genotypes and sperm concentration in infertile males (*p*-value < 0.001) as shown in Table-III.

Out of 144 patients, 80 (55.5%) patients had normal sperm motility, 57 (39.5%) patients had asthenospermia while 7 (8.4%) cases had necrospermia. Strong association was observed between NRF2 genotypes and sperm motility in infertile males (*p*-value <0.001) as shown in Table-IV.

Table-III: Association of NRF2 gene polymorphism (RS6721961) with sperm concentration in primary male infertility cases.

Genotypes	Sperm Concentration			*p*-value
	Normal Count 111 (77.0%)	Oilgospermia 29 (20.1%)	Azoospermia 4 (2.7%)	
CC	86 (77.4%)	11 (37.9%)	3 (75%)	< 0.001
CA	22 (19.8%)	10 (34.4%)	-	
AA	3 (2.7%)	8 (27.5%)	1 (25%)	

Table-IV: Association of NRF2 gene polymorphism (RS6721961) with sperm motility in primary male infertility cases.

Genotypes	Sperm Motility			*p*-value
	Normal Motility 80 (55.5%)	Asthenospermia 57 (39.5%)	Necrospermia 7 (4.8%)	
CC	71 (88.7%)	25 (43.8%)	4 (57.1%)	< 0.001
CA	7 (8.7%)	22 (38.5%)	3 (42.8%)	
AA	2 (2.5%)	10 (17.5%)	-	

DISCUSSION

From our study results we observed that single nucleotide polymorphism (RS6721961) of the NRF2 gene is significantly associated with sperm concentration and sperm motility in infertile males in the Pakistani population.

Male infertility is a complex multifactorial pathology influenced by many diseases and risk factors.[13] NRF2 is an inducible nuclear transcriptional protein that protects against the damage caused by xenobiotics and oxidative stress at the cellular level as it regulates the gene transcription and the production of associated enzymes via ARE elements that function as antioxidants.[14] In humans, most antioxidant genes, including NRF2, can develop nucleotide changes that might cause male infertility in various ways.[9]

In this study, the semen parameters of patients with primary male infertility were analyzed according to WHO guidelines (2010) for the processing and analysis of human semen and included semen volume, colour, sperm count and sperm motility. No significant association was found between NRF2 (RS6721961) genotypes and semen volume and colour of infertile males.

A strong association was found between NRF2 (RS6721961) genotypes and sperm concentration in

infertile males in the current study. These findings were consistent with the study conducted on the Chinese population, which showed NRF2 (RS6721961) AA genotype is strongly associated with oligoasthenozoospermia.[15] This result also corresponds with a study by Yu *et al*, among Chinese males who were heavy smokers. NRF2 SNP (RS6721961) AA genotype was noted to be significantly linked with a reduction in semen quality.[16] This result was also in accordance with the study conducted by Bidram *et al*, among Iranian males, which demonstrated significantly lower NRF2 expressions in smokers with reduced sperm concentration and motility.[17]

In the current study, a strong association was observed between NRF2 (RS6721961) genotypes and sperm motility in infertile males. Different studies conducted on Chinese males observed that this NRF2 SNP was strongly associated with oligoasthenozoospermia and individuals with AA genotypes had a higher risk of oligoasthenozoospermia.[15,16] A study on infertile Turkish men showed that patients with high seminal oxidation-reduction potential levels had significantly lowered their sperm count and motility under seminal oxidative stress.[18] Another recent study carried out in Islamabad, Pakistan, indicated that oxidative stress, along with a reduction in antioxidant levels, substantially lowered the fertilizing capability of infertile males.[19] This was also in agreement with the study conducted by Khalil *et al*, among infertile Egyptian males.[20]

Very little data is published in the context of genetic bases of primary male infertility in Pakistani males. Diagnosing the exact underlying etiological factors of male infertility is usually challenging and imposes a huge financial burden on the affected couples. It also sanctions a persistent negative influence on life quality. This study has analyzed the association of primary male infertility with NRF2 gene polymorphism in the Pakistani population. Early diagnosis and treatment given to infertile males can be improved by identifying the genetic risk factors associated with male infertility. Gene analysis can be introduced in the routine diagnostic testing of male infertility to improve the etiological clarification significantly. There is a need for identifying genetic risk factors to formulate a strategy to avoid irrelevant and invasive diagnostic testing of the affected couples to reduce the financial burdens and the associated anxiety. This study will also be an addition to the literature available on this subject, as limited genetic studies on primary male infertility have been done in Pakistan.

LIMITATIONS OF STUDY

We considered only one nucleotide polymorphism of the NRF2 gene, i.e. (RS6721961). Other single nucleotide polymorphisms of the NRF2 gene, i.e., rs35652124 (-653A>G), rs6706649 (-651G>A), might have a role in male infertility. We also did not evaluate the lifestyle, occupational and environmental risk factors that influence sperm quality significantly, such as cigarette smoking, alcohol consumption, caffeine intake, obesity, use of recreational drugs, psychological stress, prolonged exposure to heat, pesticides, heavy metals, inadequate Zinc and vitamin C in diet, malnutrition and anaemia etc.

RECOMMENDATIONS

Further work is required to correlate the gene expression of NRF2 genotypes and specific sperm function parameters in infertile males. Future research is needed to determine the mechanism of action of the signalling pathway of NRF2/ARE involved in the antioxidant defence system against oxidative stress in individuals affected by this gene polymorphism.

CONCLUSION

Single nucleotide polymorphism (RS6721961) of the NRF2 gene is significantly associated with sperm concentration and sperm motility in infertile males in the Pakistani population.

Conflict of Interest: None.

Authors' Contributions

BR: Curation of data, writing of original draft and statistical analysis, AR: Literary criticism and study designing, MA: Laboratory assistance and optimization of PCR, AT: Obtaining samples, HK: Counselling of patients, SQ: Reading of final manuscript.

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