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Gene Expression Profile of *eNOS* Gene in a Sample of Iraqi Asthenozoospermic Patients

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Abstract: The expression of endothelial nitric oxide synthase (*eNOS*) gene represents one of the most important causes that effect the male infertility. The eNOS is primarily responsible that create of NO in the vascular endothelium, therefore eNOS is mainly expressed in the human testicular endothelial cells, in addition of Sertoli cells and Leydig, epididymis and vas deferens which regulates the synthesis of NO. Many study informed in the first time about the NO that is synthesized by human male gamete. Experimental evidence has shown that NO in high concentrations can cause defective function of the sperm and low concentrations of NO play very important role in the control of the sperm physiology. In this study, Approximately 50 Iraqi idiopathic asthenozoospermic (AZS) patients were recruited from Kamal Al-Samraee hospital, Baghdad, Iraq during a period from 15 November 2017 to 1 May 2018 and 50 healthy men were included for this study, Gene expression for *eNOS* of reverse transcription quantitative PCR (RT-qPCR) is distinguished from other methods because of its accuracy, sensitivity. Real time PCR quantification applied in the present experiment utilized the SYBR green, a fluorescent dye which recognizes any double stranded DNA including cDNA, the amplification was recorded as a Ct value (cycle threshold). The lower Ct value indicates the presence of higher copies of the target and vice versa. In terms of gene expression, high Ct values indicate low gene expression and low Ct value indicates a high gene expression . In this study we analyzed gene expression of *eNOS* genes by RT-PCR depending Ct value to measure gene expression in AZS patients and fertile. As related with *eNOS* genes the fold of gene expression in control was lower than AZS patients 4 times. Fold number in AZS patients were 4 times for eNOS gene. In calculation of the relative expression of *eNOS* gene in all study groups , the $2^{-\Delta\Delta Ct}$ results were applied، the gene expression in fertile lower than AZS patients 4.05 times for *eNOS* gene. Fold number in fertile was 1 time for *eNOS* gene.

Keywords: Endothelial nitric oxide synthase (*eNOS*), Idiopathic asthenozoospermia AZS, Male infertility.

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Introduction

Infertility is a major health problem worldwide, affecting at least one in every eight couples, and affecting people both medically and psychosocially(1). Five to 10% of normal fertile couples take more than a year or 2 to conceive. Some couples, therefore present with a delay in conceiving purely by chance, having normal low fertility rather than subfertility. Many of these supposedly “infertile” couples will eventually conceive, even without treatment. About 6% of men between the ages of 15 and 50 years are infertile. According to the World Health Organization(2), 60–80 million couples suffer from infertility worldwide(3). A male partner

factor contributes to 40% of cases of infertility(4). In Iraq Infertility is a problem among men and women which considered as an important public health and clinical problem in Iraqis. In a study conducted in Iraq, Razzak and Wais (5) reported incidence of percentages give a strong indication of infertility among Iraqi couples. Asthenozoospermia (AZS), a disorder of sperm motility, that cause a reduced of sperm motility , a reported show at a frequency of that 1 of 5000 men have 100% immotile spermatozoa in there ejaculate and there are many Causes of AZS include metabolic deficiencies and abnormalities of the sperm flagellum(6).

Asthenozoospermia, is a cause of human male infertility and is implicated in 19% of infertile cases(7). The methods for evaluation of male infertility have typically been limited to a semen analysis measuring count, motility and morphology of the sperm. Up to 8% of infertile men have been shown to have high levels of sperm DNA fragmentation despite a normal semen analysis(8). Since sperm DNA has little capacity for repair, protection against damage is particularly important(9). The variants of the *eNOS* gene are involved in low of spermatogenesis and sperm function that be associated with man infertility in many studies of different populations like oligozoospermia and idiopathic AZS (10). The *eNOS* gene is a protein coding gene that located in the 7q35-7q36 region in chromosome 7(11), that gene contains 25 introns and 26 exons with approximately 21kb length (12,13).

Materials and Methods

Subjects and Sampling

Patients (50 asthenozoospermic) and 50 apparently healthy subjects (Control) were recruited from Kamal Al-Samraee hospital, Baghdad, Iraq. All infertile patients in this study were selected on the basis of clinical and laboratory examination. Blood samples were collected in EDTA K3 tubes from all infertile and fertile males in this study. The laboratory study was conducted in Genetic Engineering and Biotechnology Institute, university of Baghdad, Iraq during a period from 15 November 2017 to 1 May 2018.

Analysis of Gene

Genomic RNA was extracted from whole blood of infertile and fertile males using Wizard genomic DNA purification kit Geneaid (Bioneer). RNA expression was performed using two primer pairs (Table 1).

Table (1): Primers that used for RNA expression .

Primers	Sequence
Forward	GGGCTCCCTGGTATTTCCAC
Reverse	CCTAAGCTGGTAGGTGCCTG

After RNA extraction, using cDNA Extraction Kit (Geneaid) cDNA extraction, program PCR of cDNA extraction, denaturation at 37 C for 10 min, annealing 42 min, extension 5 min. The products were electrophoresed using 2% agarose gel and visualized on UV transluminator by ethidium bromide staining.

Gene Expression

Using kit (GoTaq® 1-Step RT-qPCR System (SYBR® Green). Promega. Component volumes gene expression, qPCR Master 10 µL, forward primer 2 µL, reverse primer 2 µL, template cDNA 5 µL, RNase free 6 µL, the recommended cyclic condition of PCR gene expression, was as, 40 cycles of denaturation at 95 C for 10 second, annealing at 62 C for 30 second, and extension at 72 C for 30 second. The PCR products were electrophoresed using 2% agarose gel and visualized on UV transluminator by ethidium bromide staining.

Statistical Analysis

Comparisons of genotype and allele frequencies among study groups were determined using *chi* square test by SAS user guide version (14).

Subjects

Blood and semen sample were collected from men with Asthenozoospermia patients (n=50) and fertile men as a control (n=50).

Results and Discussion

Semen Analysis

The diagnosis of male infertility is mostly based on the descriptive evaluation of human semen including the number of spermatozoa per ejaculate, sperm motility and their morphology(15). Some semen parameters of fertile men and AZS patients are present in Table (2).

No significant differences were noted between AZS patients and fertile men as related with age. The equal age allows correct comparison between the two groups as related with semen characteristic.

Semen volume was significantly ($p<0.05$) increased in fertile men when compared with AZS patients (2.66 versus 2.05 ml, respectively). No significant differences were noted in semen pH between fertile men and AZS patients group, pH values were within normal level in both two groups. Sperm count was in fertile men significantly ($p<0.01$) higher than that of AZS patients (88.20 versus 45.33 10^6/ml, respectively). In both groups, sperm count was within normal values(16). Sperm motility percentages was in AZS patients significantly ($p<0.01$) lower than that of fertile men (8.60 versus 84.10 %, respectively). Also, active sperms percentage was in AZS patients significantly ($p<0.01$) lower than that of fertile men (1.20 versus 40.15 %, respectively). The results revealed that low sperm motility percentage in AZS patients is accompanied with low active sperm percentage.

Liu and Baker (17) indicate that low sperm motility percentage led to impair male infertility. Agrawal and Said (15), observed that high percentage of progressively motile sperm in the ejaculate is critical to ensure adequate sperm transport and fertilization. The percentage of normal sperm were significantly ($p<0.01$) lower in AZS patients than in the fertile men (6.80 versus 60.50%, respectively).

In contrast, the percentage of abnormal sperm was significantly (90.05 versus 33.11 %, respectively).

Table (2): Compare between patients and fertile in seminal fluid.

Seminal parameter	Mean ± SE		P-value
	Control (No. =50)	Patients (No. =50)	
Age (year)	(20-34) 34 (35-50) 16	(20-34) 32 (35-50) 18	0.082 NS
Volume (2-2.5ml)	2.66	2.05	0.0337 *
pH (More than 7.2)	7.10	7.08	0.894 NS
Count (More than 20 x10^6/ml)	88.20	45.33	0.0001 **
Motility (More than 50%)	84.10	8.60	0.0001 **
Active (More than 5%)	40.15	1.20	0.0001 **
Nor. Sperm (More than15%)	60.50	6.80	0.0001 **
Abo. Sperm (Less than 85%)	33.11	90.05	0.0001 **

*** (P<0.05), ** (P<0.01).**

CONTROL: FERRTILE, PATIENT: ASTHENOZOOSPERMIA , NS: NON-SIGNIFICANT. **: MEANS THE DIFFERENCE IS HIGH SIGNIFICANT AT (P<0.01).

Expression for *eNOS* Genes

The expression of *eNOS* gene was determined in apparently healthy subjects and asthenozoospermic patients. Total RNA was extracted from all patients and controls.

The samples used in this study were within recommended ranges for concentration and purity. The utilization of TRIZOL in the total RNA extraction from blood sample well recommended.

Expression of *β-actin* Gene

The expression of *β-actin* gene (housekeeping gene) as a mean Ct values in apparently healthy subjects and AZS patients are presented in Table (3).

As shown from Table (3), the mean Ct values for apparently healthy subjects and AZS patients were 21.05 and 21.35 , respectively, and there were no significant difference between the two groups.

Table (3): Comparison between study groups in Ct value of *β* –actin (Mean±SE)

Group	No.	Ct value
Control	15	21.05
Patients	15	21.35
LSD		1.027 NS
P-value		0.261

CONTROL : FERTILE ,PATIENT : ASTHENOZOOSPERMIA …. NS: NON-SIGNIFICANT.

β-actin as a housekeeping genes is widely used as internal control for gene expression normalization for RT-PCR (18). Fold of *β*-actin gene expression and 2^{-Ct} values for apparently healthy subject and AZS patient are presented in Table (4).

The values of 2^{-Ct} were (0.46E-8) and (0.37E-8) for apparently healthy subject and AZS patient respectively. The fold of *β*-actin gene expression was decreased in AZS patient compared with apparently healthy subject (0.80 versus 1.00, respectively), but this result was no significant.

Table (4): Comparison of *β* – actin Fold expression between study groups.

Group	Means Ct of *β* – actin	2^{-Ct}	experimental group/ Control group	Fold of gene expression
Control	21.05	0.46E-8	0.46E-8/ 0.46E-8	1.00
Patients	21.35	0.37E-8	0.37E-8/ 0.46E-8	0.80
LSD	1.027 NS	---	---	0.216 NS
P-value	0.261	---	---	0.074

NS: Non-Significant.

Expression of *eNOS* Genes

The values of Ct, ΔCt, and $2^{-\Delta Ct}$ of *eNOS* gene for apparently healthy subjects and AZS patients are presented in Table (5).

The Ct values of *eNOS* gene were 31.74 and 30.02 for apparently healthy subjects and AZS patients, respectively. The ΔCt values of *eNOS* gene was significantly ($p<0.05$) decreased in AZS patients compared with for apparently healthy subjects (8.67 versus 10.69, respectively). In contrast, the $2^{-\Delta Ct}$ values of *eNOS* gene was significantly ($p<0.05$) increased in AZS patients compared with for apparently healthy subjects (0.0024 versus 0.0006, respectively).

Table (5): Comparison between different groups in Ct,ΔCt and $2^{-\Delta Ct}$ value (*eNOSgene*)

Group	No	Ct value	ΔCt	$2^{-\Delta Ct}$
Control	20	31.74	10.69	0.0006
Patient	40	30.02	8.67	0.0024
LSD value	---	1.481 NS	1.759 *	0.00053 *
p-value	---	0.0626	0.0337	0.0482

*** (P<0.05), ** (P<0.01).**

The results related with the fold of *eNOS* gene expression depending on $2^{-\Delta Ct}$ and $2^{-\Delta\Delta Ct}$ values are presented in Table (6).

As shown in the Table (6) and depending on the $2^{-\Delta Ct}$ method, the fold of *eNOS* gene expression was in AZS patients significantly ($p<0.01$) higher than in apparently healthy subjects (4 versus 1 fold, respectively). In addition, depending on the $2^{-\Delta\Delta Ct}$ method, the values of ΔΔCt was in AZS patients significantly ($p<0.01$) lower than those of apparently healthy subjects (-1.91versus 0.11, respectively), whereas, the value of $2^{-\Delta\Delta Ct}$ was in AZS patients significantly ($p<0.01$) higher than in apparently healthy subjects (3.758 versus 0.926, respectively). Therefore, depending on the $2^{-\Delta\Delta Ct}$ method, the fold of *eNOS* gene was in AZS patients significantly ($p<0.01$) higher than that of apparently healthy subjects (4.05 versus 1 fold, respectively).

Table (6): Fold of eNOS gene expression depending on $2^{-\Delta Ct}$ and $2^{-\Delta\Delta Ct}$methods.

Parameters	$2^{-\Delta Ct}$ method		
	Gene		P-value
	Control	Patients	
ΔCtTarget	10.69	8.67	0.0337 *
Experiment	0.0006/0.0006	0.0024 /0.0006	----
Fold of geneexpression	1	4	0.0027 **
ΔCt Calibrator	10.58	10.58	1.00 NS
ΔΔCt	0.11	-1.91	0.0064 **
$2^{-\Delta\Delta Ct}$	0.926	3.758	0.0001 **
Experiment	0.926/0.926	3.758/0.926	---
Fold of gene expression	1	4.05	0.0022 **

Control: fertile .Patient : Asthenozoospermia
NS: NON-SIGNIFICANT. **: MEANS THE DIFFERENCE IS HIGH SIGNIFICANT AT (P<0.01),

The results of this study as related with the expression of *eNOS* gene are in accordance with other previous results (19-21). Buldreghini *et al*. (19) observed a higher expression and activity of eNOS in blood leucocytes of men with idiopathic asthenozoospermia.

In addition, Song *et al*. (20) found that the mRNA level showed an obvious increase (~2.4 fold) in the semen of AZS patients.

Also, Bonanno *et al*. (21) observed that almost 50% patients with severe asthenozoospermia showed significantly increased mitochondrial DNA copy number. Song and Lewis (22) found that non-progressively motile sperm show increased mitochondrial DNA copy number. Gabriel *et al*., (23) found a negative correlation between mitochondrial DNA copy number and sperm motility in men with varicocele. Therefore, it appears necessary to detect eNOS expression levels in the semen of idiopathic asthenozoospermia patients to elucidate pathogenic role in sperm motility and function.

Combined with the results of eNOS polymorphisms in the presnt study , we can speculate that the *eNOS* gene polymorphism may enhance its transcription and the synthesis of excessive bitric oxide, ultimately inhibiting sperm quality or as a prognostic value for fertilization and embryo development (24).

Conclusion

In eNOS gene, , the fold of eNOS gene expression was in Asthenozoospermia patients 4 fold wile in apparently healthy was 1 fold.

References

1. Fisher, J.R. and Hammarberg, K. (2012). Psychological and social aspects of infertility in men: an overview of the evidence and implications for psychologically informed clinical care and future research. Asian J. Androl., 14(1): 121-129.
2. World Health Organization (WHO) (2010). Laboratory Manual for the Examination and Processing of Human Semen, 5th Edition.
3. Rutstein, S.O. and Shah, I.H. (2004). Infecundity, Infertility and Childlessness in Developing Countries. ORC Macro. MEASURE DHS+ (Programme) and World Health Organization. Calverton, MD, Geneva, Switzerland: ORC Macro World Health Organization.
4. Alam, N. (2009). Male factor infertility basics revisited. J. Coll. Physicians Surg. Pakistan, 19(4): 205 - 206.
5. Razzak, A.H. and Wais, S.A. (2002). The infertile couple: A Cohort study in Duhok, Iraq. East Mediterr Health J., 8(2-3): 234-238.
6. Ortega, C.; Verheyen, G.; Raick, D.; Camus M.; Devroey, P. and Tournaye, H. (2011).

Absoluteasthenozoospermia and ICSI: What are the options. Human Reproduction Update, 17 (5): 684-692.
7. Curi, S.M.; Ariagno, J.I.; Chenlo, P.H.; Mendeluk, G.R.; Pugliese, M.N.; Sardi L.M., *et al*. (2003). Asthenozoospermia: analysis of a large population. Arch Androl., 49(5): 343-49.
8. Sakkas, D. and Alvarez, J.G. (2010). Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome, and analysis. Fertil Steril., 93(4): 1027–1036.
9. Simon, L.; Lutton, D.; McManus, J. and Lewis, S.E. (2011). Sperm DNA damage measured by the alkaline Comet assay as an independent predictor of male infertility and IVF success. Fertil. Steril., 95(2): 652–657.
10. Faure, C.; Leveille, P.; Dupont, C.; Julia C., Chavatte-Palmer, P.; Alifert Group, *et al*. (2014). Are superoxide *dismutase* 2 and nitric oxide synthase polymorphisms associated with idiopathic infertility. Antioxid Redox Signal, 21(4): 565-569.
11. Marsden, P.A.; Schappert, K.T.; Chen, H.S.; Flowers, M.; Sundell, C.L.; Wilcox, J.N., *et al*. (1992). Molecular cloning and characterization of human endothelial nitric oxide synthase. FEBS Lett., 307(3): 287-293.
12. Gagala, J.; Buraczynska, M.; Mazurkiewicz, T. and Ksiazek, A. (2013). Endothelial nitric oxide synthase gene intron 4 polymorphism in non-traumatic osteonecrosis of the femoral head. Int. Orthop., 37(7): 1381-1385.
13. Mustafa, S.A. and Abdulwahid ,M.J. (2017). Identification of 27bp Variable Tandem Repeats in Endothelial Nitric Oxide Synthase (eNOS) Gene of Hypertensive Subjects in Kurdish Population from Erbil City. Iraqi Journal of Biotechnology, 16 (4): 1-7.
14. SAS (2004). Statistical analysis system, User's guide. Statistical Ver. 7th edition SAS. Inst. Inc. Cary USA.
15. Agrawal, A. and Said, T.M. (2011). Interpretation of Basic Semen Analysis and Advanced Semen Testing. Male Infertility. 15-22.
16. WHO (1999). Laboratory manual for the examination of human semen and sperm-cervical mucus interaction, fourth edition. Cambridge, Cambridge University Press.
17. Liu, D.Y. and Baker, H.W. (2007). Assessment of human sperm function and clinical management of male infertility. National Institutes of Health. 13(2): 99-109.
18. Lin, J. and Redies, C. (2012). Histological evidence: housekeeping genes beta-actin and GAPDH are of limited value for normalization of gene expression. Dev. Genes. Evol., 222(6): 69-76.
19. Buldreghini, E.; Hamada, A.; Macri, M.L.; Amoroso, S.; Boscaro, M.; Lenzi, A., *et al*. (2014). Human leucocytes in asthenozoospermic patients: endothelial nitric oxide synthase expression. Andrologia., 46(10): 1176–1182.
20. Song, P.; Zou, S.; Chen, T.; Chen, J.; Wang, Y.; Yang, J., *et al*. (2015). Endothelial nitric oxide synthase (eNOS) T- 786C, 4a4b, and G894T polymorphisms and male inferlity: Study for idiopathic asthenozoosper-mia and meta- analysis. Biology of Reproducon, 92(2): 38.
21. Bonanno, O.; Romeo, G.; Asero, P.; Pezzino, F.M.; Castiglione, R.; Burrello, N., *et al*. (2016). Sperm of patients with severe asthenozoospermia show biochemical, molecular and genomic alterations. *Life Sciences. 63 (2): 144-162.*
22. Song, G. and Lewis, V. (2008). Mitochondrial DNA integrity and copy number in sperm from infertile men. Fertility and Sterility, 90(6): 2238–2244.
23. Gabriel, M.S.; Chan, S.W.; Alhathal, N.; Chen, J.Z. and Zini, A. (2012). Influence of microsurgical varicocelectomy on human sperm mitochondrial DNA copy number. A pilot study. Journal of Assisted Reproduction and Genetics, 29(8): 759-764.
24. Carreau, S. and Isabelle, G.D. (2007). Transcripts of aromatase and estrogen receptors and significance of other RNAs in human spermatozoa. Arch Androl., 53(5): 249–255.