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Estimation of Estrogen Receptor Beta Gene Expression and Its Correlation with Some Hormones Parameters in Samples of Iraqi Infertile Men

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Abstract: In this study we will try to evaluate role of ERβ in idiopathic male infertility by Estimate expression level of ERβ in semen samples of infertile male compared with fertile male semen samples, Analyzed some male hormones levels (estrogen-testosterone-FSH-LH) in both infertile and fertile Iraqi men and Study the association between the gene expression of ERβ gene with hormones levels in these samples. The level of expression of *ESR2* gene in control was significantly higher than in patient group. There is non-significant positive correlation between the hormones and *ESR2* expression levels which means increase the expression level of *ESR2* will have a positive effect on those hormones while the (E2) shows a non-significant negative correlation with the expression level of *ESR2*.

Keywords: ERβ- estrogen-testosterone-FSH-LH-Infertility.

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Introduction

Infertility is defined as the inability of couples to have a child after one year of regular unprotected intercourse, affecting (10 – 15) % of couples. According to the latest WHO statistics, approximately 50– 80 million people worldwide suffer from infertility, and male factors are responsible for approximately (20– 30) % of all infertility cases (1, 2).

The human estrogen receptor beta (*ERβ*) gene or *ESR2* is located on chromosome 14 q23.2, and is ~61.2 kb. The ERβ protein is produced from eight exons. Additionally, there are two untranslated exons, 0N and 0K, in the 5′ region and an exon at the 3′ end that can be spliced to exon 7 to produce the alternative ERβ isoform (3).

The ERβ localization in germ cell, spermatozoa, epithelial cells, Sertoli and Leydig cells which suggested the important role of ERβ in spermatogenesis (4). It showed that activation of ERβ by estrogen (E2) increased proliferation of immature Sertoli cells (5). Sertoli cells are the somatic cells of the testis that are important for spermatogenesis. Sertoli cells accelerate differentiation of germ cells to spermatozoa, Sertoli cells dysfunction impairs spermatogenesis and fertility (6, 7).

Material and methods

Samples of research were collected from Kamal Al- Samara'ay IVF Hospital, Ministry of Health in Baghdad-Iraq. The time assigned for this study was extended from 1November of 2021 to end of May 2022 The study was designed as a case control study, the study was planned as

a case control study, including 100 samples of semen and blood from 75 infertile men with oligozoospermia, asthenospermia, and azoospermia and 25 fertile men as controls. Men diagnosed with varicocele and obstructive azoospermia were excluded. Samples were collected from men who abstain from sexual intercourse for 3-5 days Semen samples were collected in plastic, sterile containers then incubated at suitable condition for adequate liquefaction time then samples were subjected to microscopic examination according to WHO instructions (2010). After collecting the sample from patient between 1.5-2 ml, transfer up to 200 µl of liquid sample to a 1.5 ml of microcentrifuge tube (RNase-free) and then Add 3 volumes of GENEzol™ Reagent per 1 volume of sample (3:1) which equal to 600 µl of trizol then mix well by vortex. Incubate the sample mixture for 5 minutes at room temperature and Easy pure miRNA kit was used for extraction of miRNA gene and the extraction was done by using Spin column-based nucleic acid purification method. *ESR2* expression level was quantitatively evaluated by Real time-PCR after converting of miRNA to cDNA. Also a Three ml The serum obtained by putting the blood samples in a vacuum sterile glasses gel, serum has been collected and kept in freezer until used for hormonal assays, the Hormonal analysis of LH, FSH was performed by using Automated Immune Assay (AIA) by the VIDAS auto analyzer, (bioMérieux Company) France, while for Testosterone and E2 was Competitive-ELISA from (elabscience Company) USA.

The Statistical Analysis System-SAS (2018) [8] program was used to detect the Effect of difference factors in study parameters. Least significant difference –LSD test (Analysis of Variation-ANOVA) was used to significant compare between means. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability. Estimate of correlation coefficient between variables in this study.

Results and discussion

- **Characteristics of the subjects**

Individuals enrolled in this study were 100 individuals, 25 of them were fertile while the rest 75 individual were infertile. On the basis on sperm count and motility, patients group were subdivided into three subgroups as: asthenozoospermia which was the highest proportion subgroup involved 45% patients the rest of the cases divided into oligoasthenozoospermia subgroup and azoospermia subgroup.

- **Study group categorization**

Table (1) reveals the distribution of study groups by age. The age of patients was ranged from 21 to 49. Asthenozoospermi group was the highest proportion in patients and control groups with 45%. Age means of patients groups were as follow: (33.67 ±1.24), (31.33 ±1.85) and (32.25 ±3.67) for Asthenozoospermia, Oligoasthenozoospermia and Azoospermia respectively *versus* (30.62 ±1.64) in control group. Current results showed that age is Non- significant associated with infertility cases. This result agree with results of previous studies by Ilacqua and Izzo, 2018 which mentioned that incorrect lifestyles and environmental factors plays an important role on the decline of male fertility, particularly associated with advancing age which include different factors like stress (physical, emotional, biological, etc) can reduce the potential of male fertility, Nutritional factors indicate that high fat diets result in impaired reproduction, by affecting

molecular and physical structure of sperm as well as the health of the developing fetus and subsequent offspring. And other factor including high temperature and low physical activity which effect male sperm and lead to infertility (9).

Table (1): Comparison between Study Groups by Age

Group	Mean ± SE of Age (year)
Asthenozoospermia	33.67 ±1.24
Oligoasthenozoospermia	31.33 ±1.85
Azoospermia	32.25 ±3.67
Control	30.62 ±1.64
LSD value	0.952 NS
P-value	0.569
NS: Non-Significant.	

- **Type of infertility in patients group**

Distribution of sample study according to the type of infertility in difference groups was shown in table (2).

Table (2): Distribution of sample study according to the type of infertility in difference groups

Group	No	Primary No. (%)	Secondary No. (%)	P-value
Asthino	42	33	9 (21.43%)	0.0001 **
Oligoasthino	15	(78.57%)	1(6.67%)	0.0001 **
azo	8	14 (93.33%)	1 (12.50%) (6.67%)	0.0001 **
Total	65	7 (87.50%)	11 (16.92%)	0.0001 **
**** (P≤0.01).**				

Mean of Primary infertility in Oligoasthenozospermia was significantly higher (P≤0.01) than that of other patients group and was (78.57%, 87.50%, respectively).

Mean of secondary infertility in Asthenozospermia was significantly higher (P≤0.01) than that of other patients group and was (6.67%, 112.50%, respactivly).

The result of this study is agree with other study that mention that the number of primary infertility cases was more than secondary infertility, in men having primary infertility, genetic and chromosomal factors have a vital role in the presence of primary infertility than secondary infertility (10).

Other study has been done in Africa and the results were disagreeing with this study by showing that the proportion of primary and secondary infertility is approximately equal. And the commonest causes of male-related infertility are Oligospermia, asthenozoospermia, and varicocele. It is suggested that interpretation and utilization of these findings should consider the presence of substantial heterogeneity between the included studies (11).

The differences between the earlier research and ours could be attributed to the use of study populations from various historical eras, geographic variances, lifestyle-related characteristics, environmental factors, marital status, socioeconomic background, or semen analysis methodology.

- **Hormones analysis**

Table (3): Comparison between difference groups in Hormones

Group	Mean ± SE			
	FSH mIU/ ml	Testosterone ng/ ml	E2 pg/ml	LH mIU/ ml
Astheno	7.42 ±1.86	5.01 ±1.44 b	42.34 ±2.76 a	4.82 ±0.97
Oligoastheno	4.06 ±0.69	7.56 ±4.40 b	36.80 ±3.90 ab	3.75 ±0.43
Azo	4.45 ±1.95	22.17 ±9.30 a	32.80 ±10.42 ab	4.14 ±1.40
Control	5.87 ±0.63	7.05 ±1.38 b	26.91 ±2.19 b	4.21 ±0.44
LSD value	6.961 NS	9.888 **	13.309 *	4.25 NS
P-value	0.605	0.0127	0.0388	0.903
Means having with the different letters in same column differed significantly. * (P≤0.05), ** (P≤0.01).				

Mean level of FSH hormone in Asthinozospermia was (7.42 ±1.86) which was significantly (P≤0.01) higher than that of other patients group and control (4.06 ±0.69, 4.45 ±1.95, 5.87 ±0.63, respectively).

Mean level of Testosterone hormone in Azospermia was (22.17 ±9.30) which was significantly (P≤0.01) higher than that of other patients group and control (5.01 ±1.44, 7.56 ±4.40, 7.05 ±1.38, respectively).

Mean level of E2 hormone in Asthinozospermia was (42.34 ±2.76) which was significantly (P≤0.01) higher than that of other patients group and control (36.80 ±3.90, 32.80 ±10.42, 26.91 ±2.19, respectively).

Mean level of LH hormone in Asthinozospermia was (4.82 ±0.97) which was significantly (P≤0.01) higher than that of other patients group and control (3.75 ±0.43, 4.14 ±1.40, 4.21 ±0.44, respectively).

The result of this study show a significant difference level of FSH which is agree with study that suggest the high level of FSH may mean the testicles are not functioning correctly due to Advancing age, Damage to testicles caused by alcohol abuse, chemotherapy, or radiation ,testes don't produce enough testosterone, FSH production rises, Problems with genes such as Klinefelter syndrome ,Treatment with hormones or Certain tumors in the pituitary gland (12).

While the result of the level of Testosterone show a significant difference, which is agree with study that suggest the high level of Testosterone occur due to Tumor growth near hormonal glands, such as adrenal gland or testicles, Using anabolic steroids to build muscle mass or enhance athletic performance, Taking Testosterone supplement or Testosterone Replacement therapy (TRT) for abnormally low Testosterone levels (13).

There are significant difference in level of E2 which is agree with study that suggest the high level of E2 may occur due to Some medications and substances like (Antibiotics -herbs or other natural substances, such as gingko or ginseng - phenothiazine which is a medication used for mental health conditions) High estrogen is also could passed down through genes. other health conditions can raise estrogen levels, such as: stress-weight gain or obesity-tumors-diseases that affect the liver-conditions that affect hormone balances, such as hypogonadism (14). Particularly due to the fact that estrogen is not routinely measured in men in clinical practice. Advancements in methods to precisely measure estrogens in men, together with a reduction of their costs, should provide better evidence on this issue and inform clinical practice. New basic and clinical research is required to improve our

knowledge on the role of estrogen in male reproductive function and men's health in general.

The result show a significant difference in the level of LH which agrees with a study that explained the high level of LH occur due to several causes like testicular damage by chemotherapy, radiation, infection, or alcohol abuse. Or Klinefelter's syndrome, which is a genetic disorder that affects sexual development in males and often causes infertility. The study Also suggest that the LH might play a central role in sperm motility and normal morphology, after adjusting for various lifestyle factors and other sex hormone levels. Which supports the utility of circulating LH levels as a biomarker for assessing sperm quality (15).

Another studies that disagree with studies that mention above which revealed the Low FSH levels can have major repercussions for sperm quality. FSH deficiency can be induced by the use of exogenous testosterone, a pituitary injury or tumor. While Low testosterone could be brought on by aging or lifestyle choices. Many men with low testosterone can still generate healthy sperm because testosterone is not the hormone that truly encourages sperm production. Because of LH stimulates the production of testosterone in the testes , lack of LH can result in a lack of testosterone, which may affect fertility and sex drive (16).

While low E2 level occur due to several Causes like cardiovascular diseases , autoimmune conditions and low body weight, that could lead to beside male infertility to delayed ejaculation, coronary heart and artery diseases, delayed growth during puberty osteoporosis, insulin and depression (17, 18).

- **Quantitative expression of *Estrogen Receptor Beta (ESR2)***

The miRNA was successfully extracted from semen samples of patients groups and control group. To obtain cDNA, reverse transcription was used and the Reaction was mediated with suitable primers for *ESR2* and GAPDH as housekeeping *gene*. The annealing temperatures of the primers were 60 ºC for all genes.

Quantitative expression of *ESR2* was determined by Real Time Polymerase Chain Reaction. The gene expression was normalized to the level of a housekeeping gene (*GAPDH*) and quantified by the Δct value.

There is a significant (P≤0.05) difference in level of *ESR2* expression between patients and control group as shown in tables (4). The *ESR2* amplification plots and dissociation curves by qPCR Samples included healthy study group are shown in figure (1).

Table (4): Comparison of *ESR2* Fold expression between study groups

Group	GAPDH	Ct	ΔCt	ΔΔCt	Folding change
Control	29.02 ±0.10	29.03 ±0.10	0.613 ±0.15	-0.686 ±0.15	1.189 ±0.17
Patients	27.99 ±0.08	27.90 ±0.08	-2.87 ±0.16	-4.17 ±0.16	0.109 ±0.05
T-test	--	--	--	--	0.659 *
*** (P≤0.05).**					

The level of expression of *ESR2* in control was significantly (1.189 ±0.17) higher than in patient group.

The *ESR2* expression was detected mostly in spermatogonia, primary spermatocytes, and immature spermatids. In Sertoli cells ERβ expression increases with age. The aromatase enzyme (cP450arom), which converts androgens to estrogens, is widely expressed in human tissues (including gonads and hypothalamus), even during fetal life, suggesting that estrogens are also involved in human fetal physiology. Moreover, cP450arom is expressed in the early postnatal testicular Leydig cells and spermatogonia. Even though the aromatase complex is required for estrogen synthesis, its biological relevance is also related to the regulation of the balance between androgens and estrogens in different tissues (19).

The current study disagree with the results of Abhari *et al*., (20) which found that ERβ expression level significantly decreased in comparison with the normal group (p<0.0001).

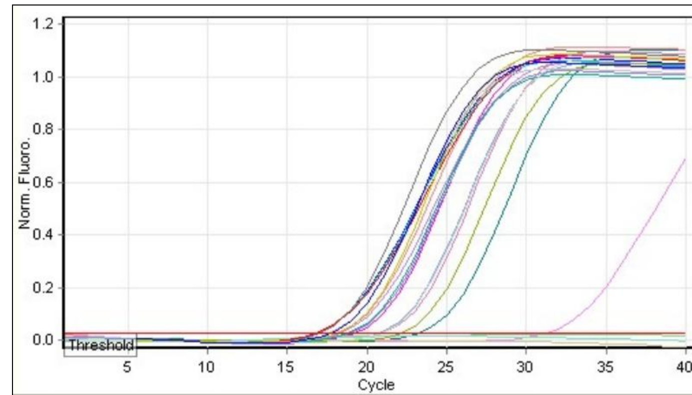

Figure (1): *ESR2* amplification plots by qPCR Samples included all study groups. The photograph was taken directly from qPCR machine.

- ***ESR2* expression level and hormones**

Table (5) show non-significant positive correlation between (FSH-Testosterone-LH) hormones and *ESR2* expression levels which means increase the expression level of *ESR2* will have a positive effect on those hormones while the (E2) shows a non-significant negative correlation with the expression level of *ESR2*.

Table (5): Correlation coefficient between *ESR-2* Gene expression and Hormones levels

Hormones	Correlation coefficient-r with Estrogen beta	P-value
FSH	0.07	0.594 NS
Testosterone	0.04	0.783 NS
E2	-0.13	0.271 NS
LH	0.09	0.502 NS
NS: Non-Significant		

The results of Abhari *et al*., (20) showed the Expression levels of ERβ were negatively correlated with E2(spearman's correlation coefficient; -0.730) (20).

During fetal and perinatal life, estrogen acts on the central nervous system by modulating the development of some areas within the brain that are committed to controlling male sexual behavior in terms of setting gender identity, sexual orientation development and the evolution of normal adult male sexual behavior. This organizational, central effect of estrogens is of particular significance in other species (especially rodents and rams), being probably less important in men where psychosocial factors become more determining. Other relevant, non-reproductive physiological events depend on estrogen in men and they involve bone maturation and mineralization as well as metabolic functions (21).

In addition, it should be remarked that the co-expression of both ERα and ERβ in the same cell determines a complex cross-talk finally resulting in the antagonistic effect exerted by ERβ on ERα-dependent transcription. Thus, the presence/absence of ER subtypes together with their cross-talk determines a cell's ability to respond to different ligands as well as the regulation of transcription of different target genes (22). Several studies suggest possible estrogen action at the level of hypothalamus. In order to clarify the role of estrogen on the feedback regulation of gonadotropin secretion at hypothalamic level (23).

Conclusion

1- The expression level of ESR2 in control is higher than patients group.
2- The mean level of LH ,FSH and E2 hormone in Asthinozospermia was higher than that of other patients group and control while the mean level of Testosterone hormone in azospermia was higher than that of other patients group and control.
3- There is a non-significant positive correlation between FSH-Testosterone-LH hormones and the ESR2 expression level which means increasing the expression level of ESR2 will have a positive effect on those hormones while the (E2) shows a non-significant negative correlation with ESR2 expression.

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