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Gene Expression of FSHR Gene in Infertile Women Undergone IVF Program

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Annotation: One of the main obstetric diseases that may cause pelvic discomfort and infertility is endometriosis (1). The link between endometriosis and fertility has long been underlined and the problem is one does not quite understand the part that endometriosis-related infertility play (2). The pregnancy and implantation rates in women who underwent endometriosis and were exposed to IVF treatment, was compared with those that were not afflicted by the problem of endometriosis (3).

Anterior pituitary gonadotropic cells synthesise folicle stimulating hormone (FSH), a gonadotropin hormone. It is crucial for reproduction, and its production is regulated by gonadotropin-releasing hormone (GnRH), which enhances biosynthesis and secretion, or inhibin, which reduces FSH production (4). It makes the body's growth, development, sexual maturity, and reproductive system work normally (5).

FSHR is a G protein couple and the G protein comprises the feature of long exodomain (7 transmembrane domain, 3 short intracellular loop, 3or extral loops and the intracellular tail). The FSH of the very large ECD binds to FSHR. It consists of molecular weight of approximately 75kDa. The amino acid sequence of protein FSHR is 695 starting with a signal peptide of 17

residues of amino acids; the complete protein consists of 678 residues of amino acids with a calculated molecular weight of approximately 75 kDa and three or four potentially glycosylated site(s) (6). A single copy gene encoding FSHR is highlighted as human FSHR gene that is located in 2p21-p16 chromosome with 54 kb length. FSHR gene in humans has a total of ten exons and nine introns together with the promoter region. The presence of other extragonadal tissues which express FSHR was found in placenta, uterus, prostate, bone tissue and the ovarian epithelia and the ovarian cancer (7). FSH combines with FSHR, which is a linked to receptor of G-protein. It can therefore be ascertained as intricate in endometriosis development by exerting its influence on the endometrial tissue by signaling cAMP through the interaction process (8). This study is in pursuit of the correlation between infertility and endometriosis to the FSHR gene expression.

Method

Patients and collection of tissue samples

The research contained 40 instances of infertile women with endometriosis (ages 23 to 38; n=20 who were getting hormone treatment and n=20 who were not). All those coming under the endometriotic were taken during laparoscopy at the Al-Yearmook Teaching hospital during the period between January 2024 and November 2024 to 2024 with an objective of trying. All lesions were always confirmed with regard to the diagnosis of endometriosis with direct visualization and histopathological specimens. During the uterine endometrial scratching operation, which was done on normal cycling patients who were getting ready for future in vitro fertilisation cycles, we got control endometrium (n = 10) from healthy women. Measurement of Hormone FSH levels were determined at the menstrual cycle in the follicular phase and so were the LH levels, levels of progesterone were determined at luteal stage of the menstrual cycle and prolactin levels were measured at luteal phase of the menstrual cycle.

Extraction of RNA and synthesis of cDNA

Following the manufacturer's instructions, we used an RNA extraction kit (Promega, USA) to get RNA from patients. We used the High-capacity cDNA kit from Applied Biosystems in the USA to turn up to 2 g of RNA into single-stranded cDNA in a 20-microliter process. This was done by first incubating the random primers for 10 minutes at 25°C, then the cDNA synthesis for 120 minutes at 37°C, and finally the enzyme for 5 minutes at 85°C to stop it from working (9).

Determine RNA yield Fluorescence Method

The quality and quantity of the isolated RNA by gel electrophoresis and NanoDrop (Thermo Scientific). To ascertain the quality of the samples to be used downstream, the concentration of the extracted cDNA using Quantus Fluorometer was determined. A 200l of diluted Quantifluor Dye was added to the cDNA of 1l of a l. The concentration of cDNA was observed after incubation at room temperature during 5 min in the dark.

Primer preparation

This company produces primers in a dry form (lyophilized) and Macrogen is a well-established biotechnology company located in Korea. Each primer was reconstituted carefully in nuclease-free water to 100 pmol/microL to make a stock solution. Subsequently, a working solution was made up by adding 10 mL of the stock solution into 90 mL of a nuclease-free water thereby causing the concentration of the final working solution to be 10 pmol/mL.

Primer name	Vol. of nuclease-free water (µl)	Concentration (pmol/µl)
FSHR_exp-F	300	100
FSHR_exp-R	300	100
β-Globin-F	300	100
β-Globin-R	300	100

These primers were despatched by Macrogen Company in the lyophilised form. Lyophilised primers were mixed with the prediluted nuclease-free water and the final concentration of the stock solution reached 100pmol/ul. To make a working solution of these primers, we mixed 10 µl of primer stock solution (kept in the freezer at -20 C) with 90 µl of nuclease-free water. This provided us with a working primer solution of 10 pmol/ 1l (10).

Gene expression

We used the Quantiflour RNA System (Promega, USA) and the Mic qPCR Cycler (Bio Molecular System, Australia) to perform RT-qPCR experiments. Macrogen (Korea) produced and standardised DNA primers that may be used to make copies of the FSHR gene.

The primer and housekeeping gene sequences are shown in Table 1. A total of 10 µl of nuclease-free water, 1 µl of forward and reverse primers (10 pmol/µl), 5 µl of 2x Master Mix, and 1 ng of cDNA were all included in the reaction mixture.

RT-qPCR cycle of FSHR gene under the following conditions; the activation of the enzyme 95c 5 min 1 r; the preset conditions of denaturation by; 95c 20s, annealing by; 65c 20s extension by; 72c 20s 40 cycles in hold 2. A melting curve was drawn following the last amplification. Relative quantification of each amplicon was done by using 2-delta delta C (11).

Table (1): Primer sequence used in gene expression

Primers	Sequence	Annealing temperature c
FSHR_exp-F	GGTTTGTCCTCACCAAGCTTCG	60
FSHR_exp-R	GGTTGGAGAACACATCTGCCTC	60
β-Globin-F	ACACAACTGTGTTCACTAGC	65
β-Globin-R	CAACTTCATCCACGTTCACC	65

Results

1. **Hormone Measurement**

Table 3-1: The mean values (±SD) of various hormone levels in patients participating in the hormone treatment program, patients not participating in the program, and the control group.

Hormones	Program with hormone therapy		Program without hormone therapy		control group	
	range	result	range	result	Normal range	units
FSH	**6.3-7.4**	**6.8**	**6.5-7.7**	**7**	**3-10**	**mIU/mL**
LH	**4.6-5.7**	**5**	**4.1-5.6**	**4.5**	**2-10**	**mIU/mL**
Progesterone	**2.6-12.7**	**9.4**	**2-7.8**	**5.8**	**2-25**	**ng/mL**
Prolactin	**10.6-16**	**13.3**	**13-17**	**15**	**4-23**	**ng/mL**
Pregnancy rate	**43.1**		**23**		**60**	**%**

In both groups, FSH and LH levels were within the normal range, with a small increase observed in the hormone-treated group. The hormone therapy group also had higher progesterone concentrations (9.4 mg/ml) than the untreated group (5.8 mg/ml), signifying that hormone therapy enhanced the hormonal environment for endometrial receptivity and effective implantation (12).

Prolactin levels were within the normal range in both groups, which excludes hyperprolactinemia as a contributing factor to infertility in this sample. The percentage was higher in pregnancy women receiving hormone therapy (43.1%) compared to untreated women (23%), while still lower than the pregnancy rate in healthy women (60%) (13).

Discussion

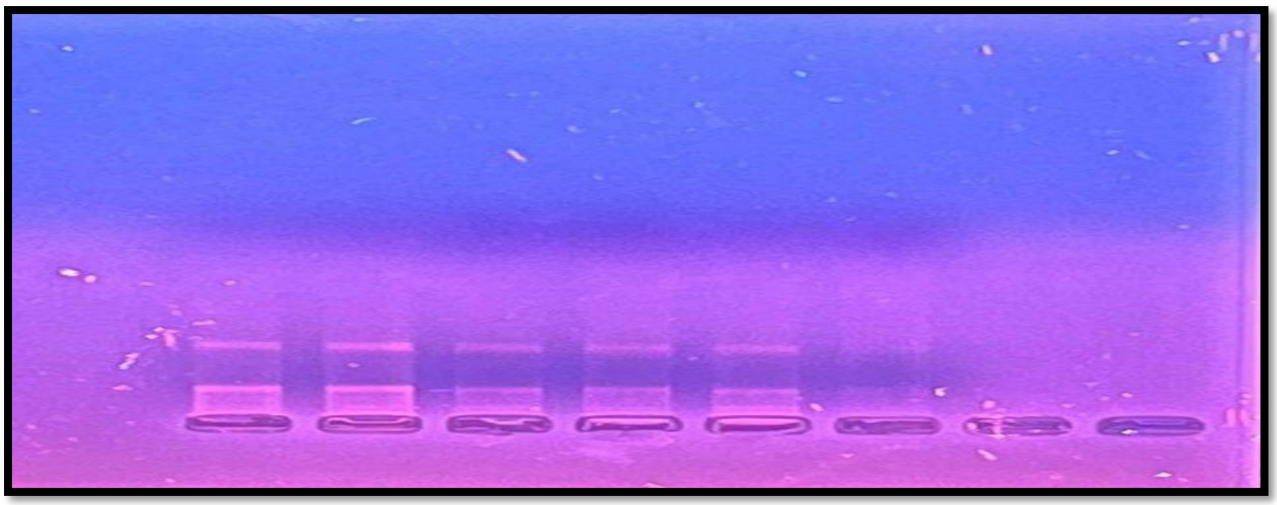
Hormonal therapy in cases of endometriosis appears to have contributed to the improvement of specific hormonal indicators like progesterone levels, which is reflected in a higher pregnancy rate compared with women not receiving treatment. Supporting the luteal phase of the menstrual cycle and encouraging early implantation depend on having high levels of progesterone. The women with endometriosis still had a notably lower pregnancy rate compared with healthy women (43.1% vs. 60%), show that endometriosis continues to impact fertility even with treatment.

In the case of FSH and LH, their levels remained within the normal range, an adequate ovarian response to treatment. the fact that the pregnancy rate has not returned to normal levels, effected on the that other factors, such as changes to the endometrial lining or immune system-related mechanisms, may contribute to decreased fertility in women with endometriosis.

Which mean that prolactin levels were normal, it can be deduced that prolactin is unlikely to be a direct cause of infertility in this group, with greater attention warranted toward the quality of the endometrium and the oocyte's competence as potential contributing factors.

2.Gel electrophoresis

Fig. (3-1) shows the result of gel electrophoresis for some samples of the FSHR gene in patients undergoing hormone therapy.



Gel electrophoresis inveterate the integrity of RNA samples as shown in Fig. (3-1), with clear 28S and 18S rRNA bands detected in all samples. The agarose gel electrophoresis method is a popular technique for determining RNA quality, and the findings are often used as a benchmark for subsequent applications such as reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and RNA sequencing. The ability to extract high-quality RNA is essential for these applications, as it ensures that the resulting data is accurate and reliable. In addition to the presence of distinct 28S and 18S bands (14). The agarose gel electrophoresis result presented in the image serves as a means of evaluating the integrity of total RNA extracted from endometrial tissues. In a standard RNA quality assessment process, the presence of two sharp bands corresponding to 28S and 18S rRNA, characteristic of eukaryotic cells, is indicative of high-quality RNA (15). Specifically, the 28S band should exhibit a higher intensity, approximately twice that of the 18S band. The image States that most lanes exhibit defined bands with consistent size and intensity, and minimal spreading. For example, the presence of a third band corresponding to the 5.8S rRNA can indicate that the RNA is of high quality, it appeared the RNA has not undergone significant degradation(16).

Discussion

The RNA integrity evident in the gel image corroborates the results from the Nanodrop purity analysis (A260/280 ~ 2.0). In conjunction, these outcomes validate that the extracted RNA was of high quality, unaffected by significant degradation, and compatible with downstream processes such as cDNA synthesis and qPCR.

This integrity is critical for the reliable analysis of FSHR gene expression, as degraded RNA can lead to the Incomplete reverse transcription, Low amplification efficiency and Misleading gene expression quantification.

The quality assurance provided by both Nanodrop and gel electrophoresis validates the experimental workflow and ensures the accuracy of FSHR mRNA quantification. This strengthens the overall conclusions drawn from your study regarding the association between endometriosis-related infertility and FSHR expression levels.

3. Nanodrop

A Nanodrop spectrophotometer was used to assess the quality of the extracted RNA by measuring the absorbance ratio at 260 and 280 nm (A260/A280).This ratio is widely accepted for RNA purity, with a value of approximately 2.0 signifying highly pure RNA that it appears free from protein contamination (17). The A260/A280 ratio values obtained for the RNA samples in this study were close to the ideal value of 2.0, indicating that the RNA was of high purity and suitable for subsequent applications such as cDNA synthesis and RT-qPCR analysis. In addition to the A260/A280 ratio, other methods such as capillary electrophoresis and quantitative PCR (qPCR) can also be used to evaluate RNA quality (18). These methods provide more information on RNA integrity and can help to find potential sources of contamination. In conclusion, the quality of the extracted RNA was evaluated using a Nanodrop spectrophotometer. The absence of considerable protein contamination ensured the accuracy and reliability of the gene expression measurements (19).

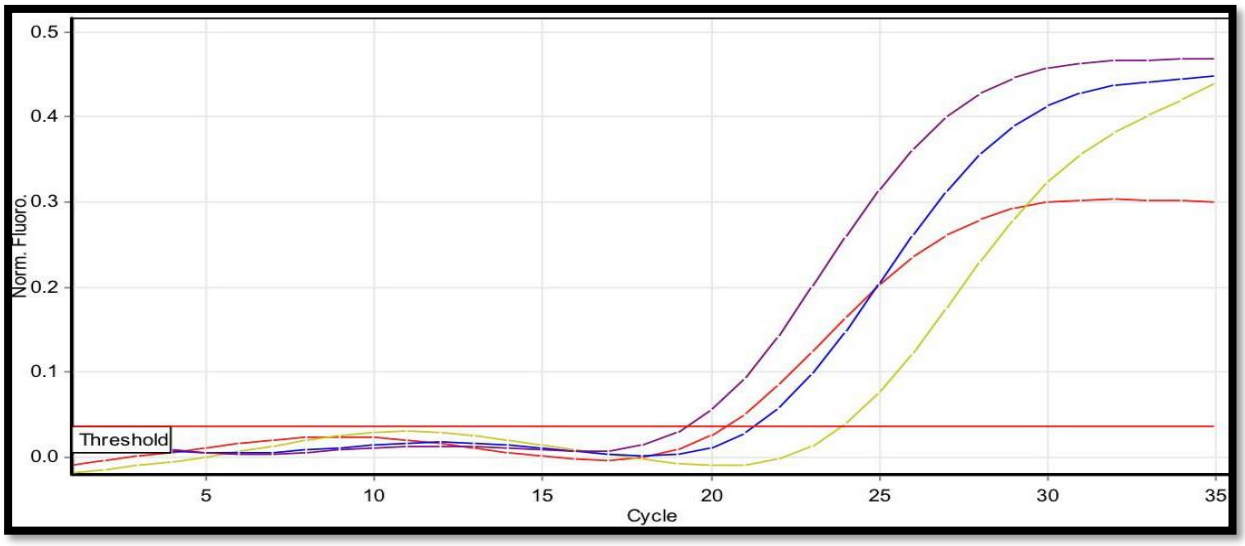
Discussion

The 260/280 absorbance ratio is a widely accepted indicator of RNA purity, particularly regarding contamination with proteins or phenolic compounds. A ratio close to 2.0 typically reflects high-quality, protein-free RNA, which is critical for reliable molecular applications such as reverse transcription and quantitative PCR (RT-qPCR).

In this study, the observed A260/A280 ratios were near to the perfect range, indicating that the RNA extracted from the endometrial tissue samples was of sufficient purity. The purity of RNA is particularly crucial in gene expression studies involving RT-qPCR, as impurities can lead to inconsistent cDNA synthesis or false quantification of gene targets. The satisfactory Nanodrop results in this research support the validity and reliability of the downstream FSHR gene expression analysis and enhance the overall credibility of the molecular findings in this study. Also, the consistent purity across samples implies that the RNA extraction protocol was efficient and reproducible, strengthening the methodological foundation of the research.

4. **RT-PCR**

Fig. (3-2) FSHR gene expression in hormone-treated endometriosis samples using RT-qPCR



RT-qPCR amplification curves proved variable FSHR gene expression across the group with hormone-treated samples (Fig. 3-2). The figure depicts amplification curves for the FSHR gene in RNA samples derived from the endometrial tissue of women with endometriosis who suffered hormonal therapy. Each curve represents a distinct sample, with the amplification signal (normalized fluorescence) increasing with each cycle, thereby indicating the presence and quantity of FSHR mRNA (20). The expression levels of the FSHR gene are diverse among the samples, with those exhibiting lower Ct values (earlier crossing of the threshold line) displaying higher expression, whereas those with higher Ct values indicated lower expression (21). In this figure, the purple and blue curves cross the threshold earlier (around cycle 20-23), suggesting strong FSHR expression. In contrast, the yellow curve appear as moderate expression, although the red curve crosses the threshold later, suggesting lower expression levels. The findings of this research outlined the possible involvement of FSHR in pathogenesis of endometriosis and significance of hormonal treatment in controlling the expression (22).

These results suggest a variable but detectable expression of FSHR among hormone-treated endometriosis samples. The earlier Ct values in some samples may reflect a positive response to hormonal therapy, enhancing the expression of FSHR, which is crucial for follicular development and endometrial receptivity. However, the variability in the amplification patterns could be attributed to individual differences in hormonal responsiveness, Heterogeneity in the scratch severity or tissue type and Potential RNA integrity differences or technical variations.

5. Statistical analysis

Table (3-2) FSHR gene expression by the treatment and control groups

Group	Sample ID	Ct value	Relative expression ($2^{-\Delta ct}$)	interpretation
Hormones treated	H1	21.5	0.0028	High expression
	H2	22.3	0.0022	High expression
	H3	23.0	0.0019	Moderate-high
	H4	24.2	0.0013	Moderate
	H5	25.0	0.0010	Moderate
Control	U1	27.8	0.003	Low expression
	U2	28.2	0.002	Very low expression
	U3	29.0	0.00015	Very low expression

Note: expression values ($2^{-\Delta ct}$) are relative to a housekeeping gene such as β-globin.

The hormone-treated group displayed lower Ct values (average ~23) than the untreated group (average ~28). In qPCR, a lower Ct value reflects a higher initial amount of target mRNA, which in this case is the FSHR gene. With group get hormonal treated displayed an average Ct value 23, then the control group have an average value 28. The relative gene expression, calculated using the $2^{-\Delta Ct}$ method, showed that FSHR expression in the hormone-treated group was approximately 8 to 15 times higher than that in the untreated group. This difference strongly implies a biological effect of hormonal therapy on gene regulation.

Discussion

These findings suggest that hormonal therapy significantly enhances the expression of the FSHR gene, which plays a critical role in ovarian follicular development and reproductive hormonal signaling. Increased FSHR expression may increase endometrial receptivity and ovarian responsiveness to FSH, thereby improving the chances of ovulation, fertilization, and implantation in women affected by endometriosis.

Despite the increased expression of FSHR, factors such as endometrial inflammation, immune responses, or anatomical distortions could still hinder the complete recovery of fertility, which may account for the lower pregnancy rate observed in the treated women compared with the healthy controls.

Statistical significance (optional)

To confirm whether the observed difference in FSHR expression between the groups was statistically significant, an independent t-test (if data were normally distributed) or a Mann-Whitney U test (for non-parametric data) could be applied.

Yielding a p-value less than 0.05 would confirm the significance of the difference in expression levels between the treated and untreated groups, thereby reinforcing the notion that hormonal therapy exerts a genuine biological effect on FSHR gene expression and potentially on fertility outcomes.

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