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Interferon-gamma Expression Profile as Diagnostic Signatures of Unexplained Infertility in Female Patients Suffer from Hashimoto's Thyroiditis

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ABSTRACT

Diagnosis of unexplained infertility (UEI) is made by exclusion and a relatively common problem that affects couples worldwide. Unfortunately, it is a not uncommon for females to suffer from Hashimoto's thyroiditis (HT). Interferon-gamma (IFN- γ) has a central key role in HT and in the ability to conceive. We aimed to estimate serum IFN- γ level and its expression profile in Egyptian women with HT and assess their possible association with UEI.

In this study, we examined 120 women with HT. We evaluated fertility in all patients; female patients who suffer from UEI were detected. Diagnosis of HT was based on the clinical data and the laboratory measures, enzyme-linked immunosorbent assay was used to measure serum IFN-γ, and the expression of IFN-γ messenger ribonucleic acid (mRNA) was assayed by real-time polymerase chain reaction (PCR).

According to the results of this study, 37.5 % of the studied females who suffered from HT were diagnosed with UEI. The serum level of IFN-γ and its gene expression showed a significant positive correlation with thyroid-stimulating hormone (TSH) and thyroid autoantibodies. However, a negative correlation was found with anti-müllerian hormone (AMH), free T4 (FT3), and free T4 (FT4). Analysis by linear regression revealed that TSH and FT3 were associated with serum level of IFN-γ; while FT3 was associated with *IFN-γ* gene expression.

We concluded that both are valued markers in diagnosing UEI in female patients suffering from HT.

Keywords: Hashimoto disease; Infertility; Interferon-gamma; Thyroiditis

INTRODUCTION

Autoimmune Hashimoto thyroiditis (HT) disease affects about 3% of the population. It is highly prevalent among women of reproductive age and negatively affects their fertility. Patients who suffer

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from HT have abnormal sex hormone metabolism and abnormal ovulation. During pregnancy, the incidence of pregnancy losses increases. Some studies found that 47.0% of patients with HT may have infertility.[1,2]

Infertility is the inability to get pregnant after a year of unprotected intercourse. It is estimated that 7%–15% of couples during reproductive age suffer from infertility. Many factors, including immunological and endocrine, might predispose to infertility. Unexplained infertility (UEI) is a frustrating state; involvement of the immune system in unexplained infertility has been found.[3]

It is known that the induction of immunologic tolerance in the decidua is vital to accept fetal alloantigens. Cytokines are crucial in establishing and maintenance of such tolerance. T helper type 1 (Th1) cytokines, e.g. interleukin (IL)-2, interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α) are thought to predispose to pregnancy failure. In contrast, T helper 2 (Th2) cytokines (for example, IL-4, IL-5, and IL-10, due to their ability to suppress Th1 responses) are responsible for fetal tolerance and the continuation of the pregnancy. Cytokine network imbalance can thus lead to unexplained infertility (UEI).[4]

Published studies highlighted that diminished fertility might result from the dysregulated immune system found in patients suffering from autoimmunity.[5,6] Disturbed balance of pro-inflammatory and anti-inflammatory cytokines is believed to have a critical role in the induction of autoimmune thyroid diseases. Additionally, the correlation between a high level of IFN- γ and autoimmune thyroiditis has been widely established.[7,8]

Therefore, we aimed to evaluate the level of serum level of IFN- γ and the expression of the *IFN- γ* gene in Egyptian women who suffered from HT and their possible associations with UEI. Accordingly, we can use serum levels of IFN- γ and *IFN- γ* gene expression as immunological markers for UEI in female patients suffering from HT.

MATERIALS AND METHODS

One hundred and twenty women with HT were included in this cross-sectional study. Women with HT were divided into fertile women (n=75) and women with unexplained infertility (n=45). We recruited patients from outpatient clinics of the Obstetrics and Gynecology and Endocrinology Unit Departments. A complete history taking and a thorough clinical examination were performed for all participants. The criterion for diagnosing thyroid dysfunction based on thyroid function tests includes positive serum antibodies, thyroid peroxidase (TPO) Ab, and/or thyroglobulin (Tg) Ab. This study obtained the approval of the ethical committee of the Faculty of Medicine, Zagazig University. The ethical approval code is #6521-18-11-2020. All participants signed a written informed consent form.

Inability to get pregnant after a year of regular unprotected intercourse is needed to define infertility and initiate infertility investigations. The duration is reduced to 6 months when the female age is above 35 years. The standard infertility evaluation as determined by the American Society for Reproductive Medicine (ASRM) practice includes a semen analysis, a hysterosalpingogram, an assessment of ovulation, and an evaluation of ovarian reserve, and a laparoscopy. To confirm the diagnosis of unexplained infertility, all tests for routine infertility evaluation should be normal. Transvaginal ultrasound (TVS) was used to evaluate the volume of ovaries and the count of antral follicular (AFC).[9]

In this study, we excluded patients with a history of states that increase androgen (such as androgen-secreting tumors, adrenal hyperplasia, 21-hydroxylase deficiency, or Cushing's syndrome), hypertension, a history of myocardial infarction, angina, stroke, pregnancy, diabetes, liver, or kidney diseases.

Blood Sampling and Testing

We collected blood samples from patients while they are between three to six days of the menstrual cycle; also, they were instructed to fast overnight. Blood samples were divided into two tubes: one ml of whole blood put into heparin-containing tubes for RNA extraction. The remaining four ml were put into plain evacuated tubes for sera separation. Sera were stored at-20^{o}C until analysis.

Assay of Thyroid Function and Autoantibody Levels

The serum levels of free T3 (FT3), free T4 (FT4), thyrotrophin (TSH), pituitary hormones like testosterone, follicular stimulating hormone (FSH), luteinizing hormone (LH), prolactin, anti-mullerian hormone (AMH), estradiol, and progesterone were measured by electrochemiluminescence on Cobas 6000 analyzer (ROCHE DIAGNOSTICS, USA).

Serum anti-TPO was assayed using the Accu-Bind ELISA kit (Monobind Inc., USA) and serum anti-Tg; using the Accu-Bind ELISA kit supplied from (Monobind Inc., USA).

IFN- γ Assay: mRNA and Protein Levels IFN- γ Gene Expression: RNA Isolation, cDNA Preparation and Quantitative Real-time Polymerase Chain Reaction (RT-PCR)

IFN- γ Gene Expression was evaluated in the blood samples of 120 women with HT. 75 of the included women were fertile, while 45 of them were diagnosed by having UEI. For this purpose, we used an RNA purification kit (Jena Bioscience, Germany) to extract RNA from the patient's blood. Producers were done according to the manufacturer's protocol. For the reverse transcription process, the SCRIPT Reverse Transcriptase kit (Jena Bioscience, Germany) was added to produce the first-strand cDNA using Oligo (dT) primer and M-MLV RT enzyme. In ice, we mix, 10 µL RNA template, 1.5 µL RNase-free water, and 1 µL Oligo-(dT) primer together, then 1 µL dNTP Mix, 4 µL SCRIPT RT buffer, 1 µL RNase inhibitor, 0.5 µL SCRIPT reverse transcriptases, and 1 µL Dithiothreitol stock solution was added. Incubation was done for 10 min at 30°C and 60 min at 50°C. Until analysis, the cDNA was stored at -20°C.

For quantitative real-time PCR, a Stratagene Mx3005P qPCR System (Agilent Tech., Germany) was used. The PCR mixture included 10 µL qPCR Green Master (Jena Bioscience, Germany), 0.5 µL of the forward primer (10 µM), and 0.5 ML of the reverse primer (10 µM) (Table 1), 5 µL template cDNA and 4 µL PCR RNase free water to have a final volume of 20 µL. The cycling program was as following, 95ºC for 10 min, followed by 40 cycles (95ºC for 15 secs, 58ºC for 1 min). The transcription levels of the β-actin gene were used as the reference gene. To obtain the normalized quantity of the target gene, the cycle threshold (CT) for the β-actin was subtracted from that of the target gene (ΔCT sample). Similarly, for controls, the ΔCT control was calculated. Then, ΔΔCT equals ΔCT sample – ΔCT control. Finally, to obtain the relative expression, we used the equation (2−ΔΔCT).

Serum IFN- γ Assay

IFN- γ secretion was measured in the serum samples of the patients and controls using human IFN-γ ELISA Kit (R&D Systems, USA), Assay Range: 15.6 - 1,000 pg/mL and sensitivity 8 pg/mL.

Statistical Analysis

For statistical analyses, the Statistical Package for the Social Sciences for Windows (version 21.0; SPSS, Chicago, IL, USA) was used. For descriptive statistics (mean±standard deviation) were used to express data. We used sample t-test (t) for parametric variables and Mann–Whitney test (z) for nonparametric variables for data analysis. Receiver operating characteristic (ROC) analysis was done to determine the probable diagnostic accuracy of serum and expression levels of IFN- γ. *p* was considered significant when<0.05 at a 95% confidence interval (CI).

RESULTS

Of the 120 women with HT studied cases, 75 women were fertile, their mean age: 12.2±2.6 years, and 45 women with UEI, their mean age: 13.2±1.9 years. The age of menarche, TSH, anti-TPO, anti-Tg were statistically significant increases in women with UEI compared to the fertile group ($p<0.001$). On the other hand, values of AMH and AFC there were significantly less in the UEI group ($p<0.001$) (Table 2).

Our findings showed that serum IFN- γ (pg/mL) level was significantly higher in women with UEI (33.9±8.6) in comparison to the fertile group (22.04±6.6), $p<0.001$. Also, the circulating *IFN- γ* gene expression level was significantly higher in women with UEI (4.02±0.56) in comparison to the fertile group (2.4±0.46), ($p<0.001$) (Figure 1).

Table 1. Primer used for reverse transcription-polymerase chain reaction (RT-PCR) for *interferon-gamma (IFN-γ)* gene

	Forward primer	Reverse primer
IFN-γ gene	5' -GCATCCAAAAGAGTGTGGAG-3'	5' -GACAGTTCAGCCATCACTTGG-3'
β-actin	5′-TTG CCG ACA GGA TGC AGA A-3′	5′-GCC GAT CCA CAC GGAGTA CT-3′

Table 2. Clinical and laboratory characteristics of women included in the study

Parameters	Fertile group (n=75)	The group with unexplained infertility(n=45)	*p*
age	26.23±9.6	29±10.46	0.056
Age of menarche	12.2±2.6	13.2±1.9	**0<0.001***
Duration of infertility	-	8.4±1.9	-
Tg (mg/dL)	148.78±2.15	150.8±3.97	0.379
FT3 (pg/mL)	2.32±0.25	1.41±0.2416	**0<0.001***
FT4 (ng/dL)	1.42±0.21	0.52±0.25	**0<0.001***
TSH (μIU/mL)	7.48±2.82	9.57±3.01	**0<0.001***
Anti TPO (IU/mL)	235.2±38.2	342.4±56.4	**0<0.001***
Anti Tg (IU/mL)	3.2±0.6	5.63±0.28	**0<0.001***
Estradiol (pg/mL)	35.6±5.7	36.23±5.1	0.800
Progesterone (ng/mL)	0.86±0.09	0.87±0.07	0.831
Prolactin (ng/mL)	9.63±1.4	9.94±1.2	0.239
AFC	7.8±1.6	8.1±1.6	0.238
FSH (mIU/mL)	7.6±1.46	7.9±1.2	0.237
LH (mIU/ml)	9.2±1.6	9.4±1.2	0.157
AMH (ng/mL)	1.4±0.16	0.89±0.16	**0<0.001***

TSH: Thyroid-stimulating hormone; FT3: Free triiodothyronine. FT4: Free thyroxine; Anti Tg: Anti thyroglobulin antibodies; anti-TPO: anti-thyroid peroxidase antibodies; AFC: antral follicle cells; HT: Hashimoto thyroiditis. * $p<0.05$

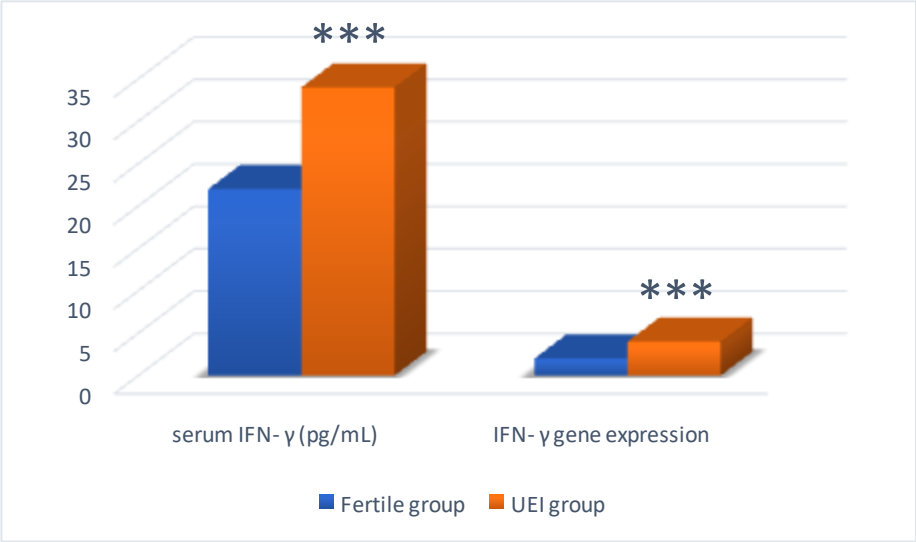

Figure 1. Comparison of serum interferon-gamma (IFN- γ) (pg/mL) and circulating *IFN- γ* gene expression level in studied groups. Subjects included 75 fertile women and 45 females with Unexplained infertility (UEI). Mann Whitney test was used for comparing means, Serum IFN- γ (pg/mL) level was significantly higher in women with UEI (33.9±8.6) in comparison to the fertile group (22.04±6.6). Circulating *IFN- γ* gene expression level was significantly higher in women with UEI (4.02±0.56) than in the fertile group (2.4±0.46). *** $p<0.001$.

To evaluate the sensitivity and specificity of the serum level of IFN- γ and the level of *IFN- γ* gene expression for diagnosis of UEI in patients of HT by ROC analysis, our results revealed that as regard serum IFN-γ, the area under the curve was 0.984 (95% CI=0.957–1.000) with sensitivity=99.6%, specificity=96%, and the cutoff values (16.7 pg/mL), (Figure 2A). Roc curve analysis showed that serum IFN-γ gene levels could differentiate fertile women from women with UEI. The area under the curve was 0.912 (95% CI=0.860–0.965) with sensitivity=90.4%, specificity=94.2%, and the cutoff values (2.65), (Figure 2B). Interestingly, the combined serum IFN- γ and *IFN- γ* gene expression results revealed that the area under the curve was 0.953 (95% CI=0.889–1.000) with sensitivity=90.4%, specificity=94.2%, (Figure 2C).

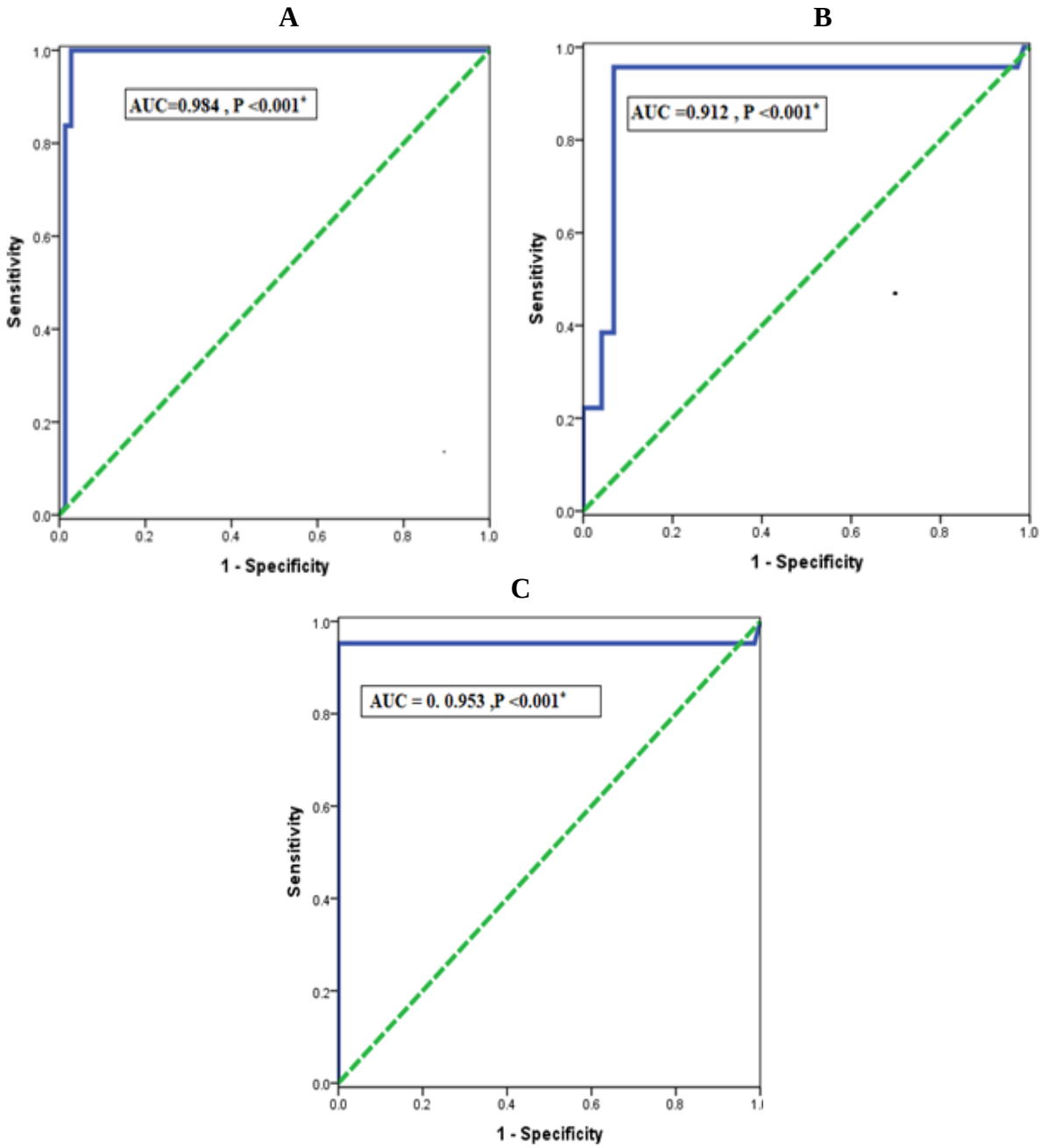

Figure 2. Receiver operating characteristic (ROC) curve of A) serum level of interferon-gamma (IFN-γ) (pg/mL), B) *interferon-gamma (IFN- γ)* gene, C) serum level of interferon-gamma (IFN- γ) for differentiating unexplained infertility (UEI) from fertile women.

DISCUSSION

Unexplained infertility is a devastating condition that greatly affects the quality of life of females who wish to conceive; The WHO estimates that infertility affects up to 50–80 million women; in 20–30% of cases, infertility remains unexplained.[10] Unfortunately, diagnosis is made by exclusion, and till now, no single test can confirm the diagnosis of such condition. Hashimoto's thyroiditis is a common autoimmune disease of females in reproductive age; 47% of affected females may have infertility without an apparent cause.[1]

IFN-γ is a key cytokine in the immunopathogenesis of HT. Gathering studies have reported that increased expression of IFN-γ results in cell-mediated immune destruction of many organs, leading to activation of humoral immune response and increased cytokine production, which leads to initiation progression of autoimmune disease. Increased levels of IFN-γ have been strongly linked to HT.[11]

Interestingly, this cross-sectional study shows that about 37.5% of females with HT in this study suffer from UEI, which is associated with idiopathic poor ovarian reserve as detected by low AFC count and AMH values in patients suffering from UIE. Other studies reported a near percentage; Quintino-Moro and colleagues reported a percentage of 47%.[1]

Thyroid hormones play important role in the sophisticated regulation of the functions of ovaries.[6] According to our results, the age of menarche and TSH was statistically significantly higher in patients with UEI than in the fertile group. Other studies had shown the influence of TSH on fertility. Jokar et al, had examined the relationship between conception rates and TSH and proposed that females with UEI have increased TSH levels than normal fertile women.[12] High level of TSH in UEI was also concluded by other studies.[13,14]

Thyroid autoantibodies play an essential role in the immunopathogenic process of autoimmune thyroiditis. Increased levels of TPO-abs are considered the most sensitive marker of autoimmune disease.[15] In this study, women with UEI showed statistically significant increases of anti-Tg and anti-TPO when compared to the fertile group. In line with our result, a study conducted by Chen et al, showed a higher rate of positive TPO-Ab in females with UEI.[16] A meta-analysis of 4 studies concluded that the presence of thyroid antibodies like anti-TPO, and anti-Tg is associated with subfertility of unexplained causes (OR 1.5, 95% CI 1.1-2.0).[17] A study by Jatzko et al. showed high anti-TPO-Ab and Tg-Ab levels were associated with failure of the in-vitro fertilization process.[18] Similarly, results confirmed by Deroux et al, showed that thyroiditis could cause infertility; even in the euthyroid state, the presence of Tg or anti-thyroperoxidase antibodies or both are related to infertility.[19]

T helper cells cytokines balance is essential to maintain fertility and success of pregnancy. Th1 pro-inflammatory cytokines, like IFN-γ, promote cell-mediated immunity. On the other hand, Th2 cells are central in humoral immunity. Th2 cytokines as IL-4, IL-5, and IL-13 regulate the activity of B cells. The Th1/Th2 cytokine balance is proposed to predict pregnancy outcomes. Multiple research groups have confirmed that successful pregnancy requires a predominant Th2-cytokine profile, while Th1-type cytokine profile is predisposed to pregnancy-related disorders.[1]

According to our results, women with UEI showed statistically significant increases in serum IFN- γ, IFN-γ mRNA compared to the fertile group. To our knowledge, this study is the first to investigate the circulating serum and expression levels of IFN- γ in Egyptian women with HT and its possible associations with clinical and laboratory characteristics of UEI as well as thyroid disease. Similar results were described in Mahdi observed that women with reproductive failure have a significant increase in serum levels of IFN- γ.[20] Also, Reid et al, confirmed higher levels of IFN- γ in infertile females.[21]

We investigated our results by ROC test to assess the power of IFN- γ serum level and the expression of *IFN- γ* gene in the diagnosis of UEI among women with HT. Regarding the power of serum level of IFN-γ in differentiating fertile women from women with UEI, the area under the curve was 0.984, a cutoff value of 16.7 mg/dL had sensitivity=99.6% and specificity=96%; concerning the power of *IFN- γ* gene expression in differentiating fertile women from women with UEF, the area under the curve was 0.912 (95% CI=0.860–0.965) with sensitivity=90.4%, specificity=94.2%. The cutoff values are 2.65 mg/dL. Regarding the power of the combination of circulating serum level of IFN-γ and the expression level of IFN-γ for differentiating UEI from fertile women, the area

under the curve was 0.953 (95% CI=0.889–1.000) with sensitivity=90.4%, specificity=94.2%. To our knowledge, this is the first study to include the trial of estimating serum level of IFN-γ and the expression level of IFN-γ as diagnostic markers of UEI in women with HT.

In conclusion, of the studied population, 37.5% of females with HT suffered from UEI. There were statistically significant increases in the age of menarche, TSH, anti-TPO, anti-Tg, serum IFN- γ, IFN-γ mRNA in the UEI group compared to the fertile females' group. Cutoff values of 16.7 and 2.65 pg/dL for IFN- γ and *IFN-γ* gene expression are specific and sensitive for differentiating UEI from fertile women.

CONFLICT OF INTEREST

All the authors declare no conflict of interest.

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