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Measuring gene expression and immune level of FOXP3 and its relationship with regulatory T cells in maintaining immune tolerance in both women pregnant and recurrent miscarriage, who suffer from autoimmune thyroid diseases

N. M. Najeeb, S. N. Mohsen, H. A. Omear
College of Science, Tikrit University, Saladin, Iraq

Autoimmune thyroid diseases (AITD) is the most common human autoimmune disease. It was found that one of the causes of its occurrence is an imbalance of the immune system of regulatory T (Treg) cells and a decrease in their transcription factor FOXP3.
The objective: **to investigate the relationship between Treg cells and FOXP3 in persons with AITD and to measure the gene expression level of FOXP3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in pregnant women and women with recurrent pregnancy loss who have AITD.**
Materials and methods**. 90 women were examined. Among them 70 patients with autoimmune thyroid diseases and elevated levels of antibodies to thyroperoxidase were divided into 35 pregnant women and 35 women with a history of recurrent miscarriages. The control group included 20 healthy women without AITD. All women were tested for FOXP3 protein levels and FOXP3 and GAPDH gene expression.**
Results**. The results showed a highly statistically significant difference in the level of regulatory T cells ($p \leq 0.001$) between the indicators of pregnant patients with AITD and women with recurrent miscarriage and AITD. The mean value in both groups was 1.7791 ± 0.4344 ng/ml and 0.9420 ± 0.1028 ng/ml, respectively.**
Highly statistically significant difference ($p \leq 0.001$) in the levels of FOXP3 protein between pregnant patients with AITD and patients with recurrent miscarriage and AITD was determined. The mean value of FOXP3 protein level was 1.3639 ± 0.2199 ng/ml and 0.7389 ± 0.2009 ng/ml, respectively. FOXP3 gene expression had significant differences between groups ($p \leq 0.001$) – it was higher in patients with autoimmune thyroid diseases (pregnant women and women with recurrent miscarriage) compared to the control group. The mean value was 2.538 ± 0.347 and 1.056 ± 0.231, respectively.
Conclusions**. The results demonstrated a relationship between the levels of regulatory T cells, and the level of FOXP3 protein and autoimmune thyroid diseases and their outcome in cases of recurrent miscarriages. It was found that the expression of the FOXP3 gene was higher in patients with autoimmune thyroid diseases (pregnant women and women with recurrent miscarriages) compared to healthy women.**
Keywords: *regulatory T cells, FOXP3, recurrent miscarriage.*

Визначення експресії гена та рівня білка FOXP3 та їхнього зв'язку з регуляторними Т-клітинами в підтримці імунної толерантності у вагітних та у жінок зі звичним викиднем, які страждають на аутоімунні захворювання щитоподібної залози

N. M. Najeeb, S. N. Mohsen, H. A. Omear

Аутоімунні розлади щитоподібної залози (ЩЗ) – найпоширеніше аутоімунне захворювання. Установлено, що однією з причин їхнього виникнення є дисбаланс імунної системи регуляторних Т-клітин і зниження в них вмісту фактора транскрипції FOXP3.
Мета дослідження: визначення зв'язку між регуляторними Т-клітинами і рівнем білка FOXP3 в осіб з аутоімунними захворюваннями ЩЗ, а також рівня експресії генів FOXP3 і GAPDH у вагітних та у жінок зі звичним викиднем, які страждають на аутоімунні захворювання ЩЗ.
Матеріали та методи. Проведено обстеження 90 жінок. З них 70 хворих з аутоімунними захворюваннями ЩЗ та підвищеним рівнем антитіл до тиреопероксидази, які були розділені на 35 вагітних і 35 жінок зі звичними викиднями в анамнезі. До групи контролю увійшли 20 здорових жінок без аутоімунних захворювань ЩЗ. Усім жінкам визначали рівень білка FOXP3 та експресії генів FOXP3 та GAPDH.
Результати. Результати продемонстрували високу статистично значущу різницю в рівні регуляторних Т-клітин ($p \leq 0{,}001$) при порівнянні показників вагітних пацієнток з аутоімунними захворюваннями ЩЗ та жінок зі звичним викиднем та аутоімунними захворюваннями ЩЗ. Середнє значення в обох групах становило відповідно 1,7791 ± 0,4344 нг/мл та 0,9420±0,1028 нг/мл.
Виявлено високостатистично значущі відмінності ($p \leq 0{,}001$) у рівнях білка FOXP3 при порівнянні показників вагітних пацієнток з аутоімунними захворюваннями ЩЗ і пацієнток зі звичним викиднем та аутоімунними захворюваннями ЩЗ. Середнє значення рівня білка FOXP3 становило відповідно 1,3639 ± 0,2199 нг/мл та 0,7389 ± 0,2009 нг/мл. Експресія гена FOXP3 мала

значні відмінності між групами ($p \leq 0,001$) – була вищою у пацієнток із аутоімунними захворюваннями ЩЗ (вагітні та жінки зі звичним викиднем) порівняно з контрольною групою. Середнє значення становило відповідно 2,538 ± 0,347 та 1,056 ± 0,231. ***Висновки.*** Отримані результати продемонстрували взаємозв'язок між рівнями регуляторних Т-клітин, рівнем білка FOXP3 і аутоімунними захворюваннями щитоподібної залози (ЩЗ) та їхнім випливом у випадках звичних викиднів. Установлено, що експресія гена FOXP3 була вищою у пацієнток з аутоімунними захворюваннями ЩЗ (вагітні і жінки зі звичними викиднями) порівняно зі здоровими жінками.
Ключові слова: *регуляторні Т-клітини, FOXP3, звичний викидень.*

Autoimmune diseases, especially hypothyroidism, affect about 5% of the population [1] and affect women 11% more than men [2]. AITD also affects pregnant women, with 15–65% of non-pregnant women having a thyroid-stimulating hormone (TSH) level significantly higher than usual [3, 4]. One of the most common autoimmune thyroid diseases is anti-thyroid peroxidase antibody (anti-TPO Ab) positive, which has a significant impact on women during pregnancy, as it leads to recurrent miscarriage (RM) [5], premature birth or infertility [6], delayed growth of the fetus in the womb when the level of antibodies rises to a large extent [7] or the fetus is born dead.

Autoimmune disorders occur as a result of various complex interactions [8]. However, the most important causes are the interactions of Treg cells, the cytokines they secrete, and the immune system's weakness when their levels decrease [9]. Treg cells protect the embryo from rejection and lead to a successful pregnancy by providing a suitable environment for the embryo's growth and inhibiting the immune cells that help in rejection [10].

For these cells to function, they must be expressed at a high level throughout pregnancy. This is done through their transcription factor, FOXP3, which regulates the work of Treg cells and maintains immune tolerance [11] Treg cells previously referred to as suppressor T cells, are a specific group of T cells that regulate the immune system, uphold self-tolerance to antigens, and hinder the development of autoimmune diseases. Treg cells possess immunosuppressive properties and primarily inhibit or reduce the initiation and expansion of effector T cells [12].

FOXP3 (Forkhead box P3), known as scurfin, is a protein in immune system responses. A member of the FOXp3 protein superfamily, consisting of transcriptional regulators. serves several functions in embryonic development and the maintenance of adult tissues, including cell proliferation, differentiation, survival, and apoptosis [13].The FOXP3 family comprises the members FOXP1, FOXP2, FOXP3, and FOXP4. The FOXP3 protein plays a vital role in Treg cells impacted by autoimmune thyroid disorders [14].

Parathyroid hormone (PTH) is a polypeptide produced in the parathyroid gland that regulates calcium and phosphorus levels in the blood [15]. Gene expression of FOXP3: The FOXP3 gene is situated on the X chromosome's short arm (p arm) and consists of 11 coding exons and three non-coding exons. The FOXP3 is a distinct and exclusive transcriptional regulator responsible for the initiation, growth, and suppressive capacity of Treg cells (Tregs).

Abnormal FOXP3 expression in Treg cells is linked to autoimmune illnesses and might potentially cause the breakdown of immunological tolerance. Increasing the expression of FOXP3 leads to the transformation of naпve T cells into Treg cells while decreasing FOXP3 expression is associated with impaired function of immunosuppressive Treg cells [16].

The objective: investigate the relationship between Treg cells and FOXP3 and the AITD and to measure the gene expression level of FOXP3 and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) between the study samples.

MATERIALS AND METHODS

This study was conducted in Saladin Governorate from February 20, 2024, until June 20, 2024, and gene expression tests were conducted in the College of Science-University of Tikrit-Iraq laboratories.

Patient group: Patient group: 90 samples were collected for the study and all samples were from women only, they were divided into 70 patients with AITD and 20 healthy (control), then 70 patients were divided into 35 pregnant patients and 35 patients with recurrent miscarriages (all 70 patients suffer from AITD). The following tests were performed for all study samples for diagnosis: triiodothyronine (T3), thyroxine (T4), TSH, and anti-TPOA. The specialist physician diagnosed 70 patients with hypothyroidism, where the average TSH for pregnant patients was 9.67 MIU/L. The average for patients with recurrent miscarriage was 43.85 MIU/L with a positive result for anti-TPOAb for all patients (pregnant and recurrent miscarriage).

In contrast, the results of the control samples were normal TSH 2.13 MIU/L with a negative result for anti-TPOA. Blood samples were taken from pregnant women with AITD at different periods of pregnancy, and none of them had ever had a previous miscarriage. As for women who also suffered from recurrent miscarriages, blood samples were taken at different periods after the miscarriage, and none of them had become pregnant during the period of sample collection. The control group was also made up of married women who had not become pregnant during the sample collection period.

Blood Sampling: 10 ml of venous blood was drawn for all samples. The blood samples were divided into four parts: a part for conducting immune tests (Treg cells, FOXP3, anti-TpoAb), amounting to 5 ml, which was collected in gel tubes, and a part for performing physiological tests (TSH, T3, T4, PTH), amounting to 3 ml. The blood serum was separated by centrifugation at a speed of 3500 rpm for 15 minutes, and a portion for conducting tests for gene expression and RNA extraction, amounting to 250 ml of blood. They were added to a tube containing 750 ml of Trizol, and all samples were stored at -45° C.

Primers

Dr. Ahmed Abdel-Jabbar Al-Fahdawi, a professor at Anbar University, designed all primers used in this study. Macrogen Company provided these primers. Dr. Adnan conducted the Ribonucleic acid (RNA) extraction and gene expression processes in the College of Science/University of Tikrit laboratories, as shown in the Table 1.

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Table 1

Primers used in this study

Primers	Sequence 5'-3'		Product size
FOXP3 gene	F	ACCTTCCACCGTTTCCTTCT	130 bp
	R	GCCTCTTGGTTTGGTTGGT	
GAPDH gene	F	TGCCACCCAGAAGACTGTGG	129 bp
	R	TTCAGCTCAGGGATGACCTT	

Quantitative Real-Time Polymerase chain reaction PCR (qPCR)

Real-time quantitative PCR was used to identify the FOXP3 gene that was normalized by the housekeeping gene (GAPDH) in patients (pregnant and recurrent miscarriages) and healthy controls according to the protocol followed by the manufacturer Macrogen/ Korea.

RNA Extraction

RNA was extracted from the blood of all research samples according to the manufacturer's protocol Macrogen/ Korea, then converted to complementary Deoxyribonucleic acid (cDNA) using a PCR device as following steps:

Step 1: Preparation of sample and Lysis

250 µL of the blood sample was added to 750 µL of Trizol and we mixed the sample well using Vortex for 1 minute. Incubated the mixture for 5 min at room temperature (R.T).

Step 2: Protein Precipitation

Added 200 µL of the Chloroform and vortex vigorously for 1 min, then, incubated tube at R.T for 5 min. all sample tubes were placed in a centrifuge at 12,000 rpm speed for 12 minutes to precipitate insoluble particles. Transferring the supernatant to a new Eppendorf tube.

Step 3: RNA Binding

An equal amount of 100% ethanol or Isopropanol is added to the filtrate and mixed well after that, 700 µL of the mixture is transferred to the Spin Column and placed in the centrifuge for 1 minute at 10,000 rpm. We repeat the previous process until the mixture runs out. The filtrate is discarded and the Spin column is transferred to a new collection tube.

Step 4: RNA Washing 1

Added 500 µL of wash buffer 1 and then centrifuge 1 min at 11.000 rpm. the filtrate is discarded and the Spin column is transferred to a new tube.

Step 5: RNA Washing 2

Added 500 µL of wash buffer 2 and then worked centrifuge for 1 min at 13,000 rpm. The filtrate is discarded and the Spin Column is in the same 2 ml Collection Tube. Performed an additional centrifuge for 2 min at 13,000 rpm to remove ethanol residual.

Step 6: RNA Elution

The Spin column was placed in new 1.5 tubes and left at room temperature for 1 minute.100 µL of the elution solution pre-warmed to (60° C) was added to the column and left for 5 min at room temperature. Centrifuge 2 min at 10.000 rpm at room temperature discarded the column and stored extracted RNA in the air to dry.

Step 7: converted RNA to cDNA

The Master Mix used was prepared by adding 384 µl of the Master Mix to 768 of RNase-free H2O in a new tube and mixing well.10 µL of the extracted RNA was added to the conversion tubes, and then 10 µL of the prepared Master Mix was added. Then, all samples were placed in a thermostatic PCR at 42° C for 15 min to get cDNA.

Step 8 q PCR to gene expression

The master mix of qPCR for FOXP3 and GAPDH genes was prepared as follows:

Added 3 ml from cDNA, 0.5 for R & F primers, 10 ml from Ultra Sybr qPCR Mix, and 6 ml from Nuclease-free water to get 20 ml from a mixture. After that, these qPCR master mix components mentioned above and placed in qPCR plate strip tubes and mixed by vortex centrifuge for 3 minutes, then put in a Real-Time PCR system.

Data Analysis of qRT-PCR

The data results of q RT-PCR for the target and housekeeping gene were analyzed by the relative quantification of gene expression levels (Fold change) (The ΔCT Method Using a reference gene) as the following equation.

$\Delta CTP = CT\ target - CT\ GAPDH$

$\Delta CTC = CT\ control - CT\ mean\ GAPDH$

$\Delta\Delta CT = \Delta CTP - \Delta CTC$

$Folding = 2^{-\Delta\Delta CT}$

Detection of Human Immune Assays Treg cells, FOXP3, and PTH in ELISA kit

Standard solutions were prepared according to the manufacturer's Shanghai YL Biont/China protocol. 50 µl each of standard solutions and streptavidin-HRP were added to the wells of the standard solutions. 40 µl of samples and 10 µl of conjugated antibodies were added to the sample wells, then 50 µl of streptavidin-HRP was added to all sample wells. Then we covered the plate with a special cover, shook it a little for mixing, and placed it in the incubator at 37° C for 60 min. the washing solution was prepared by adding 20 ml of concentrated washing solution to 980 ml of distilled water. Then the plate cover was removed and washed, and the process was repeated five times. Then we leave the plate for one minute on the filter paper to dry. Then, 50 µl of each chromogen solution A and B were added to each well and mixed well, then incubated for 10 min at 37° C away from light. We then noticed the appearance of the blue color at different concentrations, indicating the antigen reaction and its presence. Then 50 µl of the stop solution was added to each hole to stop the reaction and we noticed that the blue color turned yellow immediately. The absorbance was measured at a wavelength of 450 nm within 10.

Data Analysis of ELISA

Statistics were conducted for all research samples by Dr. Shaker M. Saleh, a lecturer at Tikrit University/ College of Agriculture, using the statistical program One-Way ANOVA.

RESULTS AND DISCUSSION

T regulatory cells

The comparison between patients (pregnant and recurrent miscarriages) all with AITD and healthy (control) in the levels of Treg cells. We found significant differences at $p< 0.005$ between them The mean and SD are in Table 2. While the comparison between pregnant and recurrent miscarriage patients (all persons with AITD). There were significant differences at $p \leq 0.001$, the mean and SD in Table 3.

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Table 2

Comparison between patients (pregnant women and women with recurrent miscarriage) with AITD and controls in Treg cell levels

Treg cell, ng/ml	Mean ±SD		P value *
	Patients	Controls	
	No = 70	No = 20	
	1.3065 ± 0.322980	1.4058 ± 0.3480	≤ 0.005

Table 3

Comparison between patients (pregnant women and women with recurrent miscarriage) with AITD in Treg cell levels

Treg cell, ng/ml	Mean ±SD		P value **
	Pregnant women	Women with recurrent miscarriage	
	No = 35	No = 35	
	1.7791 ± 0.4344	0.9420 ± 0.1028	≤ 0.001

Table 4

Comparison between patients (pregnant women and women with recurrent miscarriage) with AITD and controls in FOXP3 level

FOXP3, ng/ml	MEAN ± SD		P value *
	Patients	Controls	
	No = 70	No = 20	
	1.051 ± 0.213939	1.3949 ± 0.2255	≤ 0.005

Table 5

Comparison between patients (pregnant women and women with recurrent miscarriage) all with AITD in FOXP3 level

FOXP3, ng/ml	MEAN ± SD		P value **
	Pregnant	Recurrent miscarriage	
	No = 35	No = 35	
	1.3639 ± 0.2199	0.7389 ± 0.2009	≤ 0.001

Table 6

Comparison of CT values in patients (pregnant women and women with recurrent miscarriage) and controls for GAPDH gene expression

CT (GAPDH)	Patients, No = 70	Controls, No = 20	P value
Mean ± SD	20.751±0.514	20.493±0.612	0.226

Table 7

FOXP3 fold gene expression

Groups	Mean CT of FOXP3	Mean ΔCT of FOXP3	Folding
Control, No = 20	18.1	1.678	1.056
Patients, No = 70	26.87	1.87	2.538

Table 8

Fold change of FOXP3 expression depending on 2- ΔΔCT

Group	Means CT of FOXP3	Means CT of GAPDH	Means ΔCT	ΔΔCT	2-ΔΔCT = fold
Control, No = 20	18.1	20.493	1.678	0.000	1.056
Patients, No = 70	26.87	20.751	1.87	0.19	2.538

Table 9

Compares patients (pregnant women and women with recurrent miscarriage) with AITD and control in FOXP3 gene expression

Fold of FOXP3 gene	Mean ± SD		P value
	Patients	Controls	
	No = 70	No = 20	
	2.538 ±0.347	1.056 ± 0.231	≤ 0.001**

Forkhead3 box protein (FOXP3)

The results of the comparison between patients (pregnant women and women with miscarriages) and controls were significant at $p \leq 0.005$, and the mean and SD values are in Table 4. However, in the comparison between patients (pregnant women and women with recurrent miscarriage), the results were highly significant differences at $p \leq 0.001$. The mean and SD are in Table 5.

Real-Time PCR Quantification of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Expression

When studying gene expression, it is important to normalize the data using an internal control gene that is stably expressed to obtain accurate and reliable results. Therefore, housekeeping genes were used as an internal control gene, which is more suitable for use in studying gene expression, as it is a gene that is expressed under experimental conditions in tissues [16]. The CT value of the housekeeping gene is shown in Table 6, which compares the CT values of the housekeeping gene in both control and patient samples in the following table.

Table 6 shows no significant differences between them in the housekeeping gene expression, indicating that the endogenous gene can be expressed at a constant level in the cells and tissues of all study samples.

Real-Time PCR Quantification of FOXP3 Gene Expression

The CT values for the FOXP3 gene between the patients (pregnant women and women with recurrent miscarriage) with AITD and the controls were compared and their results appeared in Table 7.

The CT value of the FOXP3 gene is shown in Table 7. It was shown that there is a significant increase in the gene expression of FOXP3 in patients (pregnant women and women with recurrent miscarriage) l with AITD. Table 8 also shows the changes in the CT values of FOXP3 up to the fold. By conducting the mean for each stage, we found that all the results indicate significant differences, as the gene expression is higher in patients with AITD.

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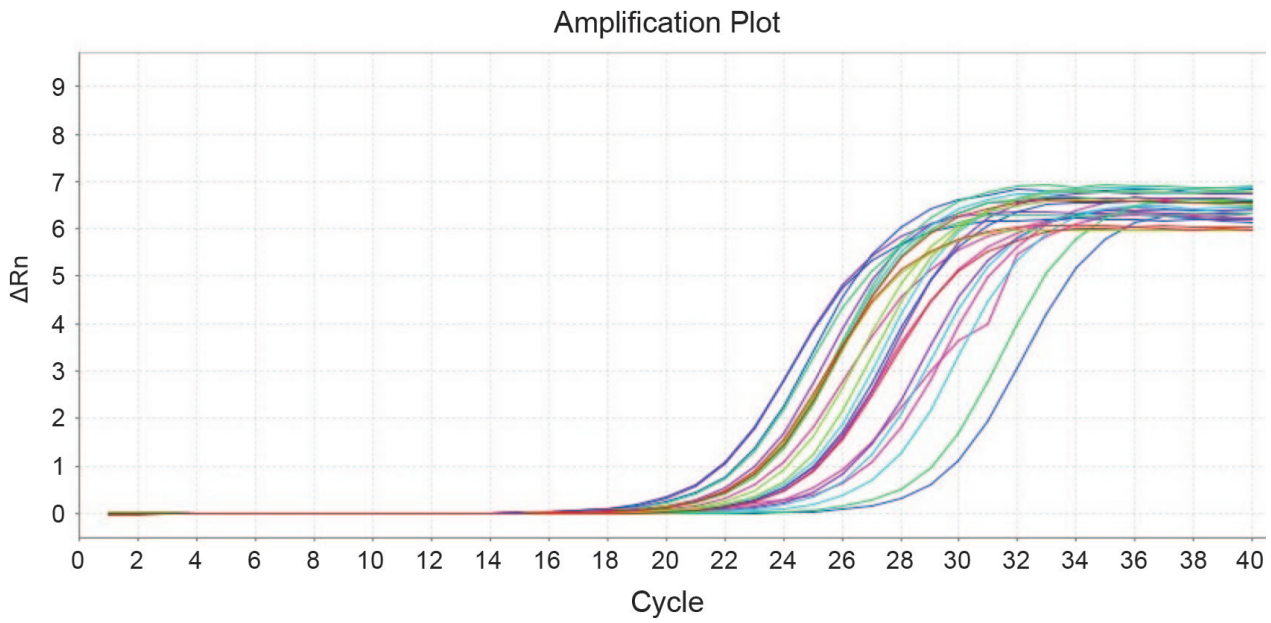

Fig. 1. FOXP3 amplification plots by qPCR samples included all study groups. The photograph was taken directly from the bio systems7500 qPCR machine. (The colors in the diagram above indicate the dilutions of the standard solutions based on which the presence of RNA in the samples is measured. The amplification starts from cycle 20 and continues to cycle 40)

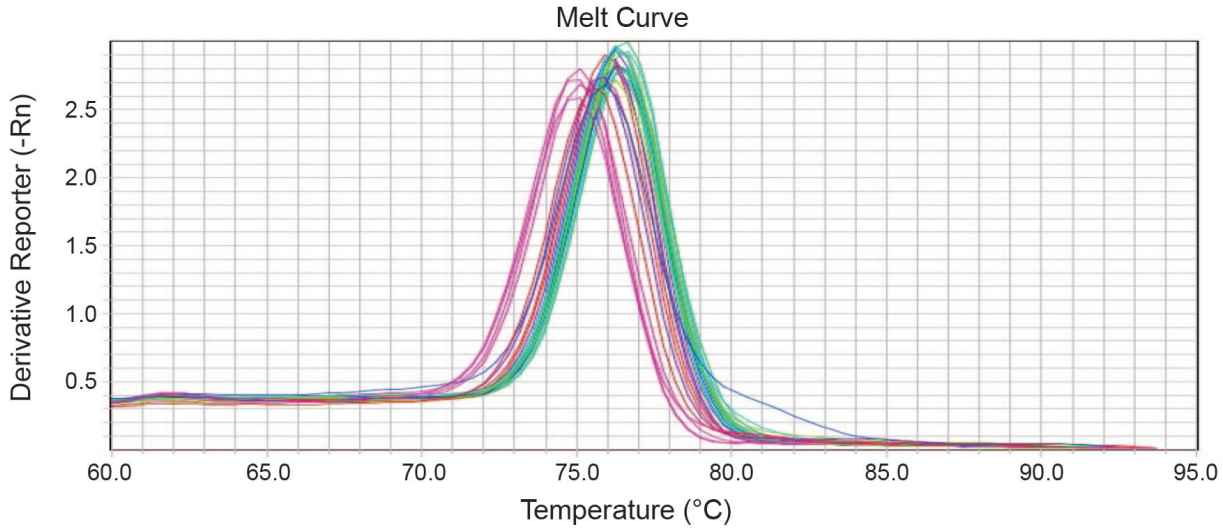

Fig. 2. FOXP3 melt curve by qPCR included all study groups. The photograph was taken directly from the bio systems7500 qPCR machine. (The diagram indicates the compatibility of the designed primers with the RNA sample present in the samples, through the matching of the curve of the primers with the samples)

By comparing the value of the fold, we find significant differences at $p \leq 0.001$ in gene expression in patients (pregnant and recurrent miscarriage) all with AITD compared to control, according to the following Table 9.

Also, when comparing between patients (pregnant and recurrent miscarriage), we found an increase in gene expression in pregnant patients at $p < 0.001$, as shown in Table 10.

Parathyroid hormone (PTH)

It is clear from Table 11, which includes a comparison between the study groups in the concentrations of PTH in blood serum, that we did not find significant differences between the groups.

Table 10

Compares patients (pregnant women and women with recurrent miscarriage) with AITD in FOXP3 gene expression

Fold of FOXP3 gene	Mean ± SD		P value
	Pregnant women	Women with recurrent miscarriage	
	No = 35	No = 35	
	0.750 ± 0.208	0.371 ± 0.112	≤ 0.001**

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Table 11

Compares between groups in PTH levels in blood serum

Group	PTH - pg/ml Mean ± SD	P value
Patients (pregnant women and women with miscarriage) with AITD, No = 70	0.1402 ± 0.126294	≤ 0.969
Control (healthy), No = 20	0.1326 ± 0.0262	
Pregnant women with AITD, No = 35	0.1413 ± 0.1471	
Women with miscarriage and with AITD, No = 35	0.1391 ± 0.1371	

Table 3 shows that there are highly significant differences at $p \leq 0.001$ between patients (pregnant women and women with recurrent miscarriages) who all suffer from AITD. These results are consistent with Logiodice F, et al.and Tang C, et al. which showed that low levels of Treg cell concentrations in patients suffering from miscarriages due to high concentrations of interleukin (IL-17) lead to increased concentrations of T-helper (Th17) cells involved in increasing miscarriage rates, as they stimulate the secretion of inflammatory cytokines [18, 19].

Our results are consistent with Idali F, et al. which states that an imbalance between Treg cells and Th17 cells leads to fetal loss [20]. It was also found that the effect of female sex hormones (estrogen, progesterone) has a significant effect in neutralizing the mother's immunity against the fetus Perkhulyn O. M. et al. [21].

So it was found that high levels of these hormones greatly support the immune tolerance of the mother and fetus, and it was found that with the increase of these hormones, the ability to support regulatory T cells and the binding of receptors specific to estrogen, especially alpha, increases the expression of FOXP3, and this indicates that a normal pregnancy in which the level of hormones is normal has a large number of regulatory cells, unlike patients who suffer from recurrent miscarriages, in which a low level of these hormones was proven, and also even after treatment with progesterone, miscarriage occurs due to hormone deficiency, and this result was consistent with both Raghupathy R,et al. and Graham J, et al. [22, 23].

It was also found that the level of the Human chorionic gonadotropin (hCG) hormone gradually decreases after the eleventh week of pregnancy, and this affects the activity of regulatory cells, as their concentration decreases. The activity of T- T-helper (TH1) cells and the inflammatory cytokines (interferon IFN-γ, tumor necrosis factor TNF-α) they secrete increase, leading to miscarriage [24].

Table 4 indicates that there are significant differences between patients (pregnant women and women with recurrent miscarriage) with AITD and controls at a significant level of $p \leq 0.005$. This is because FOXP3 is a transcription factor specific to Treg cells, which are the only ones capable of secreting and expressing it. Therefore, we find from Table 2 that the concentrations of Treg cells are increased in controls, and this is the reason for the increase in FOXP3 concentrations in controls as well Qin S, et al. [25].

As for Table 5, which indicates significant differences between patients (pregnant women and women with recurrent miscarriage) with AITD, the reason for this is that during pregnancy the need for regulatory cells increases to regulate immune tolerance between the mother and the fetus. Therefore, their number increases, especially when the concentrations of TGF-β and IL-2 increase, which work to increase the differentiation of T CD4 cells into Treg cells. Thus, FOXP3 concentrations increase.

These results agree with Deng Z, et al. [26]. As for patients who have recurrent miscarriages and AITD, the concentrations of Treg cells decrease, which leads to a decrease in the concentrations of FOXP3. It was found that the reason for this is that when transforming growth factor-β (TGF-β) is present with IL-6, this leads to the differentiation of T CD4 cells into TH17 cells, which secrete IL-17, which has a great relationship with the increase in the rate of miscarriage Luo L, et al. and Grover P,et al. supported the results [27, 28].

Table 9 the expression level of the transcription factor FOXP3 is an important factor in regulating the immune balance. Therefore, when a genetic defect occurs in this gene, this leads to a defect in the immune system and the occurrence of endocrine disorders, the most important of which are AITD of the thyroid gland. Therefore, the gene expression increased in the study samples of patients. Also, thyroid diseases are caused by a defect in chromosome XP11.23, which is the same chromosome on which the FOXP3 gene is located. These results are consistent with Bossowski A, et al. and Bacchetta R, et al. [29, 30].

Table 10 shows a comparison of the FOLD values for FOXP3 gene expression, which showed that gene expression was lower in patients with recurrent miscarriages(and with AITD) and that this was due to Signals from Treg cell receptors TCR stimulate the transcription of the FOXP33 gene, so when these signals are decreased, the expression of the FOXP3 gene is reduced. These results were supported by Colamatteo A, et al. [31].

In humans, there is a Signal Transducer and Activator of Transcription (STAT)-5 proteins (STAT5b) factor, which is a crucial transcription factor for the expression of FOXP3. When this factor is lost due to addition or deletion mutations, it loses its function, and thus, the expression of FOXp3 decreases. The Treg cells lose their function in regulation and immune tolerance. These results are consistent with Hardtke-Wolenski M, et al. and Smith M, et al. [32, 33].

As for *Table 11*, which includes the PTH results, the reason for the absence of significant differences was that the

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study samples were taking nutritional supplements containing calcium and phosphorus, and therefore the PTH levels were stable in all study samples Marcucci G, et al. [34].

CONCLUSIONS

The results of our study indicate that autoimmune thyroid diseases affect the levels of Treg cells and FOXP3 in patients (pregnant women and women with recurrent miscarriage), and their effect was clear in causing recurrent miscarriages. We also found an increase in the gene expression of FOXP3 in the patients (pregnant women and women with recurrent miscarriage) with AITD compared to the control, and we did not find clear differences in the levels of PTH between the patients (pregnant women and women with recurrent miscarriage) and control.

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Information about the authors

Najeeb Nadia Mohamed – PhD-Student, Biology Department, College of Science, Tikrit University, Tikrit, Iraq; tel.: +964 780 819 0963. *E-mail: nadia.mahameed01@gmail.com*
ORCID: 0009-0004-4236-6761

Mohsen Saria Najee – Professor, Dr. in Physiology, Biology Department- Zoology, College of Science, Tikrit University, Tikrit, Iraq; tel.: +964 771 067 1270. *E-mail: Saria7052@gmail.com*
ORCID: 0009-0001-5516-4083

Omear Hadeel Abed Alhadee – Assist Professor, Dr. in Molecular Genetics, Biology Department-Zoology, College of Science, Tikrit University, Tikrit, Iraq; tel.: +964 770 171 8651. *E-mail: hadeel.omear@tu.edu.iq*
ORCID: 0000-0003-2030-6181

REFERENCES

1. Lee HJ, Li CW, Hammerstad SS, Stefan M, Tomer Y. Immunogenetics of autoimmune thyroid diseases: A comprehensive review. J Autoimmun. 2015;64:82-90. doi: 10.1016/j.jaut.2015.07.009.
2. Hollowell JG, Staehling NW, Flanders WD, Hannon WH, Gunter EW, Spencer CA, et al. Serum TSH, T(4), and thyroid antibodies in the United States population (1988 to 1994): National Health and Nutrition Examination Survey (NHANES III). J Clin Endocrinol Metab. 2002;87(2):489-99. doi: 10.1210/jcem.87.2.8182.
3. Blatt AJ, Nakamoto JM, Kaufman HW. National status of testing for hypothyroidism during pregnancy and postpartum. J Clin Endocrinol Metab. 2012;97(3):777-84. doi: 10.1210/jc.2011-2038.
4. Moreno-Reyes R, Glinoer D, Van Oyen H, Vandevijvere S. High prevalence of thyroid disorders in pregnant women in a mildly iodine-deficient country: a population-based study. J Clin Endocrinol Metab. 2013;98(9):3694-701. doi: 10.1210/jc.2013-2149.
5. Thangaratinam S, Tan A, Knox E, Kilby MD, Franklyn J, Coomarasamy A. Association between thyroid autoantibodies and miscarriage and preterm birth: meta-analysis of evidence. BMJ. 2011;342:d2616. doi: 10.1136/bmj.d2616.
6. Consortium on Thyroid and Pregnancy – Study Group on Preterm Birth; Korevaar TIM, Derakhshan A, Taylor PN, Meima M, Chen L, et al. Association of Thyroid Function Test Abnormalities and Thyroid Autoimmunity With Preterm Birth: A Systematic Review and Meta-analysis. JAMA. 2019;322(7):632-41. doi: 10.1001/jama.2019.10931.
7. Minaldi E, D'Andrea S, Castellini C, Martorella A, Francavilla F, Francavilla S, et al. Thyroid autoimmunity and risk of post-partum depression: a systematic review and meta-analysis of longitudinal studies. J Endocrinol Invest. 2020;43(3):271-7. doi: 10.1007/s40618-019-01120-8.
8. Beraya DYu. Women have clinical aspects of infertility with thyroid pathology. Reprod Health Woman. 2022;(7):30-4. doi: 10.30841/2708-8731.7.2022.272469.
9. Ruggeri RM, Cristani M, Vicchio TM, Alibrandi A, Giovinazzo S, Saija A, et al. Increased serum interleukin-37 (IL-37) levels correlate with oxidative stress parameters in Hashimoto's thyroiditis. J Endocrinol Invest. 2019;42(2):199-205. doi: 10.1007/s40618-018-0903-3.
10. Saito S, Shima T, Nakashima A, Inada K, Yoshino O. Role of Paternal Antigen-Specific Treg Cells in Successful Implantation. Am J Reprod Immunol. 2016;75(3):310-6. doi: 10.1111/aji.12469.
11. Bending D, Ono M. From stability to dynamics: understanding molecular mechanisms of regulatory T cells through Foxp3 transcriptional dynamics. Clin Exp Immunol. 2019;197(1):14-23. doi: 10.1111/cei.13194.
12. Hassan AM, Neinaa YM, El-Bendary AS, Zakaria SS. MicroRNA-146a and Forkhead box protein 3 expressions in nonsegmental vitiligo: an insight into disease pathogenesis. J Egyp Women's Dermatol Soc. 2019;16(2):105-11. doi: 10.4103/ JEWD.JEWD_19_19.
13. Bending D, Prieto MP, Paduraru A, Ducker C, Marzaganov E, Laviron M, et al. A timer for analyzing temporally dynamic changes in transcription during differentiation in vivo. J Cell Biol. 2018;217(8):2931-50. doi: 10.1083/jcb. 201711048.
14. Bending D, Paduraru A, Ducker CB, Prieto MP, Crompton T, Ono M. A temporally dynamic Foxp3 autoregulatory transcriptional circuit controls the effector Treg programme. EMBO J. 2018;37(16):e99013. doi: 10.15252/embj.201899013.
15. Seunghwan J. Secondary hyperparathyroidism: pathogenesis and clinical aspects. Cent Asian J Endocrinol. 2023;3(2):6-17. doi: 10.5281/zenodo.8264458.
16. Zhang Y, Zhang J, Liu H, He F, Chen A, Yang H, et al. Meta-analysis of FOXP3 gene rs3761548 and rs2232365 polymorphism and multiple sclerosis susceptibility. Medecine. 2019;98(38):e17224. doi: 10.1097/MD.0000000000017224.
17. Vaiphei ST, Keppen J, Nongrum S, Chaubey RC, Kma L, Sharan RN. Evaluation of endogenous control gene(s) for gene expression studies in human blood exposed to 60Co γ-rays ex vivo. J Radiat Res. 2015;56(1):177-85. doi: 10.1093/jrr/rru074.
18. Logiodice F, Lombardelli L, Kullolli O, Haller H, Maggi E, Rukavina D, et al. Decidual Interleukin-22-Producing CD4+ T Cells (Th17/Th0/IL-22+ and Th17/Th2/IL-22+, Th2/IL-22+, Th0/IL-22+), Which Also Produce IL-4, Are Involved in the Success of Pregnancy. Int J Mol Sci. 2019;20(2):428. doi: 10.3390/ijms20020428.
19. Tang C, Hu W. The role of Th17 and Treg cells in normal pregnancy and unexplained recurrent spontaneous abortion (URSA): New insights into immune mechanisms. Placenta. 2023;142:18-26. doi: 10.1016/j.placenta.2023.08.065.
20. Idali F, Rezaii-Nia S, Golshahi H, Fatemi R, Naderi MM, Goli LB, et al. Adoptive cell therapy with induced regulatory T cells normalises the abortion rate in abortion-prone mice. Reprod Fertil Dev. 2021;33(3):220-28. doi: 10.1071/RD20063.
21. Perkhulyn OM, Pakharenko LV, Sukhin VS, Saltovskiy OV, Kovalchuk VM, Hranovska HI, et al. Evaluation of hormonal function in women with cervical insufficiency and infertility in the history. Wiad Lek. 2021;74(10):2412-16.
22. Graham JJ, Longhi MS, Heneghan MA. T helper cell immunity in pregnancy and influence on autoimmune disease progression. J Autoimmun. 2021;121:102651. doi: 10.1016/j.jaut.2021.102651.
23. Raghupathy R, Szekeres-Bartho J. Progesterone: A Unique Hormone with Immunomodulatory Roles in Pregnancy. Int J Mol Sci. 2022;23(3):1333. doi: 10.3390/ijms23031333.
24. Huang N, Chi H, Qiao J. Role of Regulatory T Cells in Regulating Fetal-Maternal Immune Tolerance in Healthy Pregnancies and Reproductive Diseases. Front Immunol. 2020;(11):1023. doi: 10.3389/fimmu.2020.01023.
25. Qin S, Li L, Liu J, Zhang J, Xiao Q, Fan Y, et al. CD4+CD25+Foxp3+ regulatory T cells regulate immune balance in unexplained recurrent spontaneous abortion via the Toll-like receptor 4/nuclear factor-κB pathway. J Int Med Res.

2020;48(12):300060520980940. doi: 10.1177/0300060520980940.
26. Deng Z, Fan T, Xiao C, Tian H, Zheng Y, Li C, et al. TGF-β signaling in health, disease, and therapeutics. Signal Transduct Target Ther. 2024;9(1):61. doi: 10.1038/s41392-024-01764-w.
27. Luo L, Zeng X, Huang Z, Luo S, Qin L, Li S. Reduced frequency and functional defects of CD4+CD25highCD-127low/- regulatory T cells in patients with unexplained recurrent spontaneous abortion. Reprod Biol Endocrinol. 2020;18(1):62. doi: 10.1186/s12958-020-00619-7.
28. Grover P, Goel PN, Greene MI. Regulatory T Cells: Regulation of Identity and Function. Front Immunol. 2021;(12):750542. doi: 10.3389/fimmu.2021.750542.
29. Bossowski A, Borysewicz-Sańczyk H, Wawrusiewicz-Kurylonek N, Zasim A, Szalecki M, Wikiera B, et al. Analysis of chosen polymorphisms in FoxP3 gene in children and adolescents with autoimmune thyroid diseases. Autoimmunity. 2014;47(6):395-400. doi: 10.3109/08916934.2014.910767.
30. Bacchetta R, Barzaghi F, Roncarolo MG. From IPEX syndrome to FOXP3 mutation: a lesson on immune dysregulation. Ann N Y Acad Sci. 2018;1417(1):5-22. doi: 10.1111/nyas.13011.
31. Colamatteo A, Carbone F, Bruzzaniti S, Galgani M, Fusco C, Maniscalco GT, et al. Molecular Mechanisms Controlling Foxp3 Expression in Health and Autoimmunity: From Epigenetic to Post-translational Regulation. Front Immunol. 2020;(10):3136. doi: 10.3389/fimmu.2019.03136.
32. Hardtke-Wolenski M, Landwehr-Kenzel S. Tipping the balance in autoimmunity: are regulatory t cells the cause, the cure, or both? Mol Cell Pediatr. 2024;11(1):3. doi: 10.1186/s40348-024-00176-8.
33. Smith MR, Satter LRF, Vargas-Hernández A. STAT5b: A master regulator of key biological pathways. Front Immunol. 2023;(13):1025373. doi: 10.3389/fimmu. 2022.1025373.
34. Marcucci G, Altieri P, Benvenga S, Bondanelli M, Camozzi V, Cetani F, et al. Hypoparathyroidism and pseudohypoparathyroidism in pregnancy: an Italian retrospective observational study. Orphanet J Rare Dis. 2021;16(1):421. doi: 10.1186/s13023-021-02053-3.

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