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Relationship between Serum Level and Gene Expression of IL-18 in Recurrent Miscarriage Iraqi Women Infected with Toxoplasmosis

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Abstract

Infectious toxoplasmosis, caused by an obligate intracellular protozoan *Toxoplasma gondii*, is a prevalent zoonotic disease that affects humans and warm-blooded animals. Cytokines are a class of signaling molecules that are widely employed in cellular communication; cells produce them, and they have an impact on how other cells interact. The aim of this study was to clarify the relationship between serum levels and gene expression of interleukin18 in miscarried women with toxoplasmosis. A Total of 200 blood samples from patients and controls were collected from Baghdad, Iraq between November 2021 and March 2022 which were used to measure gene expression of IL-18 using real-time PCR. Enzyme-linked immunosorbent assay was used to detect the level of IL-18. The findings of this investigation showed that the IL-18 serum concentration elevated significantly in healthy pregnant women compared to women with recurrent miscarriage and toxoplasmosis, recurrent miscarriage without toxoplasmosis and healthy women. Also, women who had recurrent miscarriage with toxoplasmosis showed significant differences compared with women who had recurrent miscarriage as well as healthy women ($p< 0.005$). Additionally, significant differences between patient and control were revealed by gene expression data. Also, there was statistically positive correlation between toxoplasmosis and IL-18 gene expression in the patient group. The IL-18 gene expression provided sensitive and precise toxoplasmosis diagnosis. Summing everything it can be concluded that serum level and gene expression of IL-18 elevated in recurrent miscarriage women infected with toxoplasmosis in comparison with other studied groups. Thus, the expression of IL-18 gene could serve as an indicator for women with miscarriage and toxoplasmosis.

Keywords: Toxoplasmosis, Women with recurrent miscarriage, IL-18 gene expression.

العلاقة بين المستوى المصلي والتعبير الجيني للانترلوكين 18 في النساء العراقيات ذواتِ الأسقاط المتكرروالمصابات بداء المقوسات

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الخلاصة

داء المقوسات الكوندية هومرض يسببه طفيلي التوكسوبلازما وهو من الطفيليات الأبتدائيه التي تكون

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أجبارية المعيشة داخل الخلايا والتي تنتقل عن طريق الحيوانات التي تسبب داء المقوسات في البشر والحيوانات ذوات الدم الحار. السيتوكينات هي فئة من جزيئات الإشارة تستخدم على نطاق واسع في الاتصالات الخلوية وتنتجها الخلايا ولها تأثير على سلوك الخلايا الأخرى. كان الغرض من هذه الدراسة هو الكشف عن العلاقة بين مستويات مصل والتعبير الجيني للإنترلوكين 18 في النساء المجهضات المصابات بداء المقوسات. بين نوفمبر 2021 ومارس 2022، تم جمع 200 عينة دم من عينات المرضى وعينات مجموعة السيطره من بغداد/ العراق. تم استخدام عينات الدم لقياس التعبير الجيني باستخدام qPCR اما عن مستوى IL−18 فقد تم قياسه عن طريق مقايسة الممتز المناعي المرتبط بالإنزيم. أشارت النتائج الى أرتفاع تركيز IL−18 مع وجود فروق معنوية لدى النساء الحوامل مقارنة بالنساء المجهضات المصابات بداء المقوسات الكوندية، النساء ذوات الأسقاط المتكرروالنساء الأصحاء. بالأضافة الى ذلك النساء المجهضات المصابات بداء المقوسات كان لهن فروق ذات دلالة إحصائية مقارنة بالنساء ذوات الإسقاط المتكرر والنساء الأصحاء (p <0.005). علاوة على ذلك ، تم الكشف عن فروق ذات دلالة إحصائية بين عينات المرضى و عينات السيطرة من خلال بيانات التعبير الجيني. أيضًا ، في مجموعات المرضى ، كان هناك ارتباط إيجابي إحصائيًا بين داء المقوسات والتعبير الجيني لـ IL−18، لذلك كانت دقة التعبير الجيني لـ IL−18 حساسة ومحددة لداء المقوسات.يمكن الأستنتاج ان مستوى المصل والتعبير الجيني لـ IL−18 قد ارتفع في النساء المجضات المصابات بداء المقوسات مقارنة مع المجموعات الأخرى المدروسة، لذلك فأن التعبير عن IL−18 قد يكون بمثابة مؤشر للنساء المجهضات والمصابات بداء المقوسات.

1 - Introduction

Worldwide, toxoplasmosis, caused by *T. gondii*, is a highly neglected disease that can be fatal to both humans and animals [1, 2]. Any mammal or bird can get affected and zoonotic infection can be significantly contributed to by the latter [3]. According to research, *T. gondii* has already infected more than one-third of the world's population [4]. Transplacental passage of the parasite leads to congenital toxoplasmosis. The prevalence of congenital toxoplasmosis dramatically varies from 1 to 100 per 10,000 live births in different countries and even in different regions or communities in one country [5]. It is estimated that approximately 30–50% of the world population is infected by the *T. gondii* [6]. IL-18 concentrations are known to vary through the course of infection and are clearly dependent on the parasite strain and inoculum used. Gorfu *et al.* [7], have suggested that this cytokine plays a pivotal role in mediating acute toxoplasmosis with the cytokine playing an important early role in the control of parasite replication. However, high levels of IL-18 have previously been shown to cause dysregulated induction of prepathological cytokine levels that contribute to lethality in high-dose, virulent infections [8]. If a woman is infected with *T. gondii* while being pregnant, there is a potential for transmission of this disease affecting her fetus [9]. Serious fetal abnormalities such as hydrocephalus, mental retardation, and chorioretinitis, have been linked to infections in pregnant women, especially during the first trimester [10]. In addition to causing spontaneous abortion and premature labor, toxoplasmosis in pregnant women can lead to abortion or congenital toxoplasmosis in the fetus [11, 12]. Symptoms of congenital toxoplasmosis can include neurological or ocular issues [13].

To combat *T. gondii*, an effective immune response is vital and cytokines are instrumental in aiding this process. These proteins facilitate various immunological reactions within the body. IL-18 signaling is one crucial element of the immunodulatory cytokine network for host defense, inflammation and tissue regeneration [14]. Studies show that IL-18, a cytokine that operates through inflammasomes, may work with IL-12 to enhance IFN-responses during *T. gondii* infection, helping restrict the parasite [7]. Recently, a study by Naemah *et al.* showed an increase in macrophage nitric oxide level in patients with visceral leishmaniasis compared with the control groups [15].

Previous research revealed that IL-1 and IL-18 stimulate IFN production during *T. gondii* infection [16], thus hypothesizing that activating inflammasomes is integral to human resistance to *T. gondii*.

2. Materials and Methods

2.1. Study Subject

A total of 200 blood samples from women were collected at Al-Alawiya Maternity Teaching Hospital and AL-Yarmouk Teaching Hospital in Baghdad Iraq between November 2021 and March 2022. The two primary groups that comprised of 100 patients and 100 controls, were further divided into four subgroups. The control groups included 50 healthy pregnant women (HP) and 50 healthy non-pregnant women (HNP). The patient groups consisted of 50 women with recurrent miscarriage but no toxoplasmosis (RMWOT), and 50 women with recurrent miscarriage diagnosed with anti-*Toxoplasma* IgG and IgM (RMWT). In addition, the patient groups were divided into age according to positivity of IgG and IgM antibodies was studied.

2.2. Ethical Approval

Permission was granted by the Ministry of Health and Environment, Baghdad, Iraq, and the study was approved by the local ethics committee of the Faculty of College of Science, University of Baghdad (ref: CSE/1121/0085).

2.3. Measurement of the IL-18 Concentrations

Five ml of venous blood was withdrawn from patients and controls by venipuncture of which 3 ml was then placed in plain tubes, and was allowed to clot to be centrifuged later at 4000 rpm for 10 min. The obtained serum was stored at -80°C until use. The remaining 2 ml of blood was pipetted into EDTA-containing tubes for RNA extraction and IL-18 gene expression estimation by real-time PCR. The ELISA sandwich kit of Wuhan Fine Biotechnology was used to test the serum of the research group for IL-18 levels.

2.3.1. Quantitation of IL-18 Cytokine Transcripts by Real Time PCR

Using the protocol provided by the manufacturer, the whole blood sample was utilized to extract total RNA via the Easy Pure® Blood RNA Kit (cat no. ER401) by TransGen Biotech Company in China. Next, the commercially available TransZol Up Plus from TransGen biotech, with instructions provided by the manufacturer, was used to isolate total RNA. The reverse-transcription-quantitative time polymerase chain reaction (RTqPCR) was employed to measure IL-18 gene expression. For this, total RNA was then transcribed into complementary DNA using the EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (cDNA), the recommended reaction volume for this procedure was 20µl, and this was followed. A total of 20 microliters of RNA was required to carry out reverse transcription.
cDNA was utilized as a template alongside TransGen biotech's TransStart® Top Green qPCR Super Mix AQ131-01 to perform RT-qPCR. As per the manufacturer's instructions, forward and reverse IL-18 oligonucleotides were included, as well as primers for both directions of GAPDH, a reference gene also known as glyceraldehyde-3-phosphate dehydrogenase [17]. To create a reaction mixture with a final volume of 20 µl, 10 µl of TransStart® Top Green qPCR Super Mix (2X), 1 µl of each primer (10 mM), 2 µl of cDNA, and 6 µl of Nuclease free water were combined in line with the instructions given. And were then placed in a real-time thermocycler (MIC-4 Real-time PCR System, MX 3000PTM / StratageneL/ USA). The reaction mixture experienced the following optimized cycling conditions: A 30 second initial denaturation at 94°C, five seconds of denaturation at 94°C, 15 seconds of annealing at 60°C, 20 seconds of extension at 72°C, and one cycle of melt curve at 55 to 95°C. The fold change

in gene expression was recorded as 2 Ct, representing the target gene's relative expression levels compared to a calibrator in normal subjects. The results showed the change in expression levels of the target gene as a fold following normalization to an endogenous control (housekeeping gene).

Table 1: Primer used for RT-PCR of IL-18.

Primer	Sequences	Tm	Product Size
Forward	CACCGATTTGAACGCTTGTG	60	167
Reverse	CTTCCCGTCTTCCCTTCCTT	62	90

2.4. Statistical Analysis

In San Diego, California, USA, GraphPad SoftwareGraph Pad Prism version 8.0.1 for Windows ran the outcomes of the study. IL-18 concentration was presented as an average along with standard deviation. A one way ANOVA analysis scrutinized important differences. *p*-values less than 0.05 were considered statistically significant. The RT-PCR method detected a fold in IL-18 gene expression.

3. Result and Discussion

3.1. Serum level of IL-18 in the Studied Groups

Notably, findings regarding IL-18 revealed a noteworthy variation between pregnant females and other groups with a probability of $P<0.05$. Upon comparison with the expectant mothers who had levels of IL-18 at 116± 41.65 pg/ml, women with frequent miscarriage were accompanied by toxoplasmosis, healthy women, and women with recurring miscarriage generally displayed depleted IL-18 levels at 96.70 ± 24.04 pg/ml, 82.52 ± 10.81 pg/ml and 80.73± 25.90 pg/ml respectively (Figure 1).

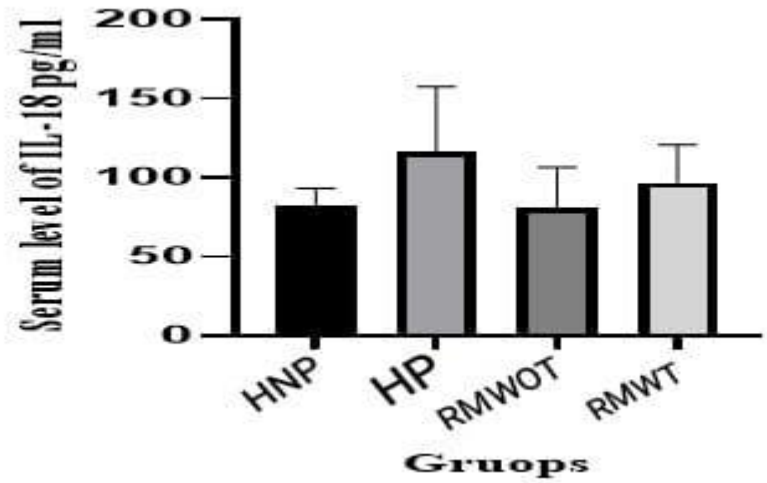

Figure 1: Comparison of serum IL-18 levels between study groups (mean ± standard deviation).

With regards to IL-18 levels in pregnant women, the findings of this study align with previous research. Specifically, AL-Khateeb [18] who reported a marked rise in serum IL-18 levels amongst pregnant individuals. Furthermore, the current study also revealed that women experiencing recurrent miscarriage with toxoplasmosis manifested an increased IL-18 concentration compared to both healthy and recurrent miscarriage women. In addition, Ida *et*

al. [19] investigated serum IL-18 levels measurement across various stages of pregnancy, including during labor, puerperium and complicated pregnancies. Another study by Bayram *et al.* [20] showed an increase of chemokine ligands (CCL2) production in patients with cutaneous leishmaniasis compared to the control groups. Notably, IL-18 levels displayed a significant increase from the first trimester until the onset of delivery in the sera of pregnant women. Once labor began, IL-18 levels saw an uptick and persistently rose to the minimum until the third day of puberty. The interplay of cytokines emitted by Th1 and Th2 cells is linked to the triumph of pregnancy [21]. Further, there is a reduction [22] associated with physiological maturation of the fetus throughout pregnancy. Production rates of cytokines hinge on hereditary factors in humans [23]. Physiological termination of pregnancy is marked by the beginning of labor, thus causing the shift in Th1/Th2 balance from a Th2 dominant state towards a Th1 dominant state. The involvement of IL-18 in pregnancy has been suggested with potential impacts on labor initiation, fetal growth restriction and pregnancy-related fatty liver. It is speculated that IL-18 might even be involved in ending pregnancies by altering the Th1/Th2 balance. One theory has suggested that successful pregnancies may be facilitated by the expression of interferon-ɣ caused by IL-18, leading to increased activation of natural killer cells (NK) that help prepare the uterus for implantation and promote vascularization. Intriguingly, it has been suggested that natural killer cells contribute to the process of 'decidualization' in early pregnancy, as evidenced by their presence in large quantities at uterine and embryonic implantation sites [24]. The crucial cytokine IL-18 has been shown to have varying concentrations depending on the strain and inoculum of the parasite during infection, indicating its significant role in controlling acute toxoplasmosis [7]. However, it is worth noting that IL-18 high levels have been linked to the development of pro-pathologic cytokines, leading to fatal consequences in cases of virulent infections [8].

3.1.1. Distribution of Toxoplasmosis Cases by Age Groups

The distribution of toxoplasmosis among different age groups according to IgM antibody positivity was also studied. The results in Figure 2 show that the prevalence of toxoplasmosis in women with miscarriage aged 26-30 was higher and was significantly higher than that in other age groups (p<0.004)

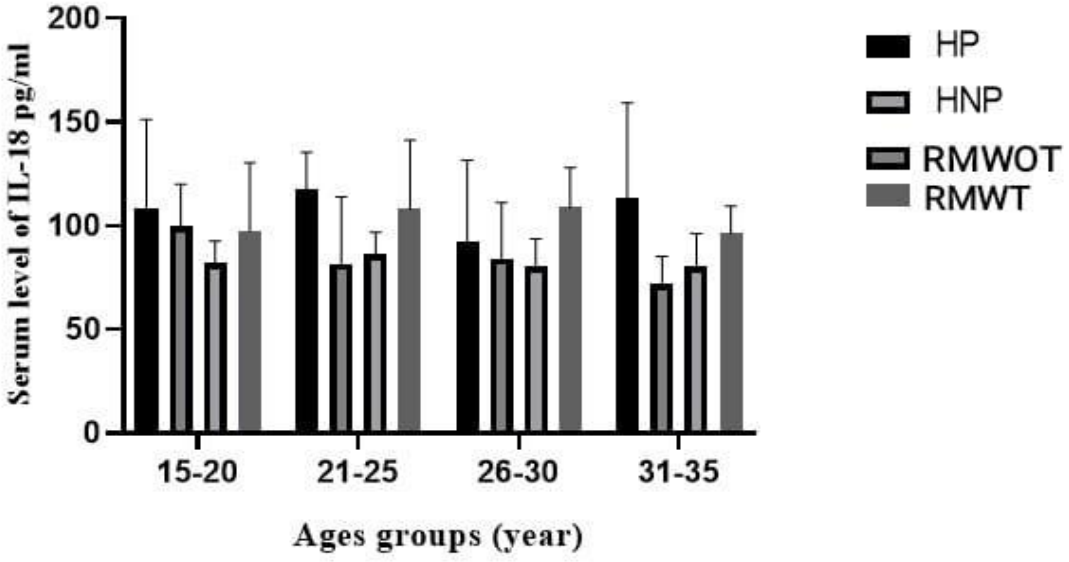

Figure 2: Distribution of toxoplasmosis according to the age.

Salih's research [25] observed that toxoplasmosis prevalence among women who suffered a

miscarriage climbed as they aged. Mohammed *et al*. [26] conducted a similar study and found that women between the ages of 26 and 30 had a higher chance of having toxoplasmosis. This observation was also supported by various other studies in different regions of Iraq that saw an increase in anti-*Toxoplasma* antibodies between ages 20-40 [27]. Further research revealed that women between the ages of 15 and 20 had the lowest likelihood of having *T. gondii* compared to other age groups. It appears that toxoplasmosis is rampant in grown women, but younger and elderly individuals are more likely to come into contact with kitties and have weaker immune systems that can't fight off *T. gondii* [28]. Its prevalence varies depending on the type of test used to diagnose it, and the host's response to different parasite strains influences specificity and sensitivity.

3.2. Gene Expression of IL-18

IL-18 expression was studied in recurrent miscarried patients with toxoplasmosis, recurrent miscarriage, pregnant women and healthy women using qRT-PCR. Analysis of mRNA expression data normalized by the GAPDH gene revealed increased IL-18 mRNA levels in toxoplasmosis-positive women compared with healthy women (Tables 2 & 3) as mean gene expression increased from 1 in healthy women to 4.5 in women with toxoplasmosis. This up regulation may be associated with a genetic polymorphism in IL-18 rs1946519, or an immune response to *Toxoplasma* infection, other studies mentioned that the G allele being associated with higher expression levels [29].

Table 2: Folding of mRNA IL-18 expression dependent on the $2^{-\Delta\Delta Ct}$ approach.

Groups	Means Ct of IL-18	Means Ct of GAPDH	ΔCt (Means Ct of *IL18*- Means Ct of GAPDH	$2^{-\Delta Ct}$	ΔΔCt	$2^{-\Delta\Delta Ct}$	Experimental Group/ Control Group	Fold of Gene Expression
RMWT	24.86	18.88	5.980	0.0158	-2.190	0.0158	0.0158/0.0035	4.563
RMWOT	26.02	18.99	7.030	0.0077	-1.140	0.0077	0.0077/0.0035	2.204
HP	25.97	19.02	6.950	0.0081	-1.220	0.0081	0.0081/0.0035	2.329
HNP	27.12	18.95	8.170	0.0035	0.000	0.0035	0.0035/0.0035	1.000

In the study done by Rajabi *et al.* [30], highly significant expressions in IL-18 levels were recorded in toxoplasmosis patients which aligns with the findings of the current study. Similarly, Suzuki *et al*. [31] discovered that the RNA expression levels for IL-18 in the brains of RAG1-/-→RAG1-/- mice were significantly greater than RAG1-/-→IFN-y -/- mice during reactivation of cerebral *T. gondii* infection. Using an acute infection model, it was demonstrated that IL-18 plays an important role in resistance against *T. gondii* in mice deficient in IL-18 and IL-18R [24]. During the acute stage of infection, IL-18 levels in sera were significantly higher in wild-type mice. A study found that caspase-1/11-deficient mice had lower serum IL-18 levels and a higher mortality rate due to increased parasite growth. Meanwhile, both IL-18-/- and IL-18R-/- mice had a higher mortality rate because of parasite proliferation [24].

Toxoplasma gondii can induce inflammation in the intestines, leading to decreased IFN-y levels in IL-18-/- mice. This also decreases intestinal necrosis in these mice following oral infection with *T. gondii* [32]. CD4+ T cells produce IFN-g which causes intestinal necrosis in mice infected orally by this parasite [33]. By working together with IL-12, IL-18 stimulates the CD8+ T cells to produce IFN-y, according to Okamoto *et al.* [34]. The RAG1-/-→RAG1-/- mice displayed notably higher levels of IL-12 expression in their brains compared to RAG1-/-→IFN-g-/- mice. Hence, in infected RAG1-/-→RAG1-/- mice, it was feasible that the brain-resident cells' IFN-y-induced upsurge in IL18 expression contributes to boosting the IFN-y production of both CD4+ and CD8+ T cells entering the brain through reactivation of cerebral *T. gondii* infection. Other studies mentioned that the infection with parasite like *Leishmania donovani* is associated with higher expression levels in patient group in comparison with the control group [35].

4 - Conclusion

This study demonstrated the role of IL-18 in the immune response to substances during infection that can lead to women miscarriage. Furthermore, an association between serum levels and gene expression was observed in the group of hospitalized IL-18 patients.

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