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Original paper

Gene expression of two innate cytokines in a miscarriage toxoplasmosis woman

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ABSTRACT. *Toxoplasma gondii* infections are endemic in Iraq and represent serious problems. Human toxoplasmosis can be associated with serious clinical manifestations, particularly in developing fetus. This study was aimed to determine the distribution of genotypes and alleles, residing within interleukin-6 (IL-6) and interleukin-1 beta (IL-1β) polymorphisms, among fetuses and neonates, congenitally infected with *Toxoplasma gondii*, and among uninfected control cases. Blood samples were collected from 125 aborted women with a history of single or recurrent miscarriage, in addition to fifty normal healthy control women. Molecular identification of the parasite was performed by detecting *Toxoplasma B1* gene using real-time qPCR technique. IL-6 and IL-1β gene expression was assayed in each case-study samples by using RT-PCR. *T. gondii* was detected in recurrent toxoplasmosis aborted women at percent (16%). IL-1β and IL-6 gene expression was significantly increased in toxoplasmosis women compared with healthy control women. Fold expression of IL-1β was 9.5 in toxoplasmosis patients compared with one fold in healthy control. IL-1β and IL-6 over-expression was correlated to the high-risk toxoplasmosis infection and could be a biomarker for prognosis of the disease.

Keywords: *Toxoplasma gondii*, abortion, RT-PCR, pro-inflammatory cytokines

Introduction

Toxoplasma gondii belongs to a vicious intracellular worm of Coccidae. It appeared three stages; tachyzoites, tissue bradyzoites and sporozoites leading to toxoplasmosis [1,2]. Primary infection of toxoplasmosis during pregnancy leads to congenital toxoplasmosis. Structural components of *T. gondii* induce chemokine production and pro-inflammatory cytokines [3]. Pregnancy potentially affected by the immune system, toxoplasmosis is one of parasitic diseases which promote a powerful Th1 reaction lead to negative effects on pregnancy [4]. Infected hosts need to detect the invasion of pathogens to prevent their spread ILCs (Innate Lymphoid Cells) are newly identified subset of lymphocytes linked to innate immunity, the ILCs are subdivided into three classes. Community 1ILCs (ILC1s) are composed of ILC1s and standard NK (cNK) cells containing Th1cytokines such as IFN-α and TNF-α [5]. Group two ILCs for example generate Th2 cytokines IL-4, IL-5, IL-9 and IL-13 and group 3ILCs (ILC3s) produce IL-17A and IL-22 and specifically express a transcriptional factor RORγt [6]. The most frequent route in *T. gondii* infection occurs through oral ingestion of *T. gondii* cysts of undercooked meat or dirty water. A primary *T. gondii* site is the intestine. ILC1s were the main producers of IFN-γ and TNF in response to the *T. gondii* oral infection [7]. As a result, the secondary immune responses against *T. gondii* infection activated macrophages which lead to unregulated production of IL-1β which involved in the pathogenesis of a variety of diseases [8]. IL-1α and IL-1β can prompt mRNA expression of hundreds of genes, act as (a positive-feedback loop) [9]. The proinflammatory cytokine formed by leukocytes, adipocytes, endothelial cells, fibroblasts, and myocytes is interleukin 6 (IL-6). IL-6 induces the manufacture of cytokine release mediators such as TNF and IL-1, which are inflammatory reaction drivers [10]. In order to

prevent exacerbation of inflammatory processes caused by proinflammatory molecules, the immune system uses anti-inflammatory appliances to escape tissue damage and restore homeostasis [11]. An imbalance between pro- and anti-inflammatory cytokines avoids the adequate function of the immune system [12]. In this study, we evaluate a possible imbalance between gene expression of pro- and anti-inflammatory cytokines (IL-6 and IL-1β) concurrently in blood samples of toxoplasmosis aborted women and the deregulation of inflammatory molecules.

Materials and Methods

Subjects

A total of 125 blood samples were collected from aborted women by venipuncture in their first visit to the women and children hospital in Al-Muthana Governorate/Iraq during the period from January to July 2019, 50 women were positive toxoplasmosis in addition to 50 healthy control women with age ranged between 20 and 40 years for both groups. Blood samples were divided into three tubes, plain tubes without anticoagulant for serum collection for determining serological biomarkers, tubes with anticoagulant for genomic DNA extraction, and tubes containing 0.75 ml of TRIzol for studying IL-1β, IL-6 gene expression of two cytokines.

Genomic DNA extraction

Genomic DNA was extracted from blood samples of aborted women by using a high pure PCR template kit (Promega, USA). DNA solution was diluted to 100 µl with elution buffer and kept at −20°C before further molecular analysis. Quantity of *T. gondii* DNA was quantified according to [15] by amplification of B1 gene by RT-PCR using specific primers: F-5'-AAGCAGCGTATTGTCGA GTAGAT-3'; R'-5-CGTCTCTTTCATTCCCACAT TTT-3' under the following thermal cycling conditions. GoTaq DNA Polymerase activation 1 cycle for 5 min at 95°C, initial denaturation for 1 cycle at 95°C for 5 min, denaturation for 40 cycle at 95°C, then primer annealing and extension for 40 cycle at 72°C for 2 min. and final extension at 72°C for 10 min. Polymerase chain reaction was performed for detection of IL-6 and IL-1β using qPCR (Favorgen, Europe) kit. DNA was amplified using a common forward primer and reverse primer shown in Table 1. The conditions for amplification were achieved as follows: initial denaturation at 95°C for 4 min followed by 35 cycles of denaturation at 95°C for 45s, annealing at 60°C for 30s, and extension at 72°C for 60s followed by a single final extension step at 72°C for 10 min. Standard curves, produced with serially diluted product size of PCR product was confirmed by electrophoresis on 2% agarose gel.

Measurement of gene expression

mRNA was extracted from different blood samples of aborted women using TRIzol™ reagent (Thermo Scientific, USA). Blood samples were kept into 0.75 ml of TRIzol™ reagent and homogenized by pipetting up and down several times. A volume of 0.2 ml of chloroform was added to the lysate, incubated for 2–3 min and spun at 12,000 rpm for 10 min. Total RNA was extracted using Favorgen kit (Europe). mRNA was converted to cDNA by using RT-PCR. Quantification of cytokine gene expression was performed according to three-shold cycle (C_t) method [15]. Gene expression of cytokines by Real Time PCR of IL-1β and IL-6 was done to determine the mRNA level according in Table 2. All of the samples were checked twice. The thermal protocol was as follows: 1 minute at 90°C, followed by 40 cycles (20 seconds at 95°C for denaturation, 20 seconds at 60°C for annealing, and 20 seconds at 72°C for elongation – when the signal was acquired at 72°C). Distilled water or cDNA synthesized in the absence of reverse transcriptase was used in place of DNA template as a negative control. cDNA was synthesized with the first strand cDNA synthesis kit (BioLab.UK)

The expression was determined by mixing the reagents including 2× Syber Green real time master mix, template RNA, primers and RNAase free water according to the manufacturer's guidelines. About 0.5 µl of RNA was added to the PCR plates and program of the machine was set according to the manufacturer's instructions. Reverse transcription for total RNA was performed using the (BioLab qPCR Master Mix) for RT-qPCR. All reactions were performed in triplicate and error bars reflect the standard deviation of these triplicates. Ct value of target mRNAs was normalized to GAPDH reference gene and the expression of mRNAs was determined by the relative quantitative method using the comparative Ct formula: Folding = 2^-ΔΔCT.

Statistical analysis

Statistical analysis system- SAS (2012) program

Table 1. Primers used for amplification of IL-1β and IL-6 gene

Cytokine	Primary sequence (5'- 3')	Tm (°C)	Product (bp)	Reference
IL-6	F: CAGAAGAACTCAGATGACTG	58	431	[13]
	R: GTGGGGCTGATTGGAAACCC			
IL-1β	F: GTTGTCATCAGACTTTGACC	59	249	[14]
	R: TTCAGTTCATATGGACCAGA			

was used. Results were expressed as mean ± standard deviation (SD). Statistical differences were evaluated using student's t-test, ($P \leq 0.05$) is considered significant and ($P < 0.01$) is highly significant.

Ethical aspects

Informed written consent of the Ministry of Health in Iraq, was obtained from all patients that accepted to participate in this study.

Results and Discussion

In this study, gene expression of IL-6 and IL-1β was assayed in miscarriage toxoplasmosis women. First of all, molecular detection of *T. gondii* in blood samples of 50 aborted women was performed by PCR analysis. Results showed that high percentage of toxoplasmosis (15.5% and 19%) was found in recurrent miscarriage and single miscarriage cases respectively. The presence of *Toxoplasma* DNA in the maternal blood probably indicates a recent infection or apparent parasitaemia which is likely to be clinically significant. The clearance time for *Toxoplasma* DNA from the blood of patients with acute toxoplasmas' lymphadenopathy was estimated to be 5.5–13 weeks [17]. These findings are similar to the results obtained by [18] who found that 14.7% were positive for *Toxoplasma* DNA in miscarriage cases.

Gene expression of IL-6 and IL-1β was assayed in the infected women compared with healthy control women (control -ve), and non-infected miscarriage women (control +ve). Results indicated in Table 3 showed that there is a significant increase ($P < 0.01$) gene of both cytokines genes in toxoplasmosis women. The intracellular parasite *T. gondii* induces the upregulation of IL-1β gene expression and release IL-1β from primary human monocytes during infection, this may be related with activate signaling for spleen tyrosine kinase (Syk), PKCδ, CARD9/MALT-1, and NF-μB that is essential for IL-1β development in primary human monocytes these findings are similar to those mentioned by [9], who found that the intracellular parasite *T. gondii* activates the release of IL-1β. Previous studies showed that pro-IL-1β mRNA expression in *T. gondii*-infected macrophages directly corresponded to the release of mature IL-1β protein. The inflammasome adaptor protein ASC

Table 2. Primers used for measurement of gene expression of IL-1β and IL-6 cytokines

Gene	Primary sequence (5'- 3')	Tm (°C)	Product (bp)	Reference
IL-6	F: CTCCTTCTCCACAAGCGCCTTC	59	583	[16]
	R: GCGCAGAATGAGATGAGTTGTC			
IL-1β	F: AAACAGATGAAGTGCTCCTTCCAGG	62	391	[16]
	R: TGGAGAACACCACTTGTTGCTCCA			
GAPDH	F: AGAAGGCTG GGGCTCATT TG			NCBI
Housekeeping gene	R: AGG GGCCAT CCA CAG TCTTC			

Table 3. Fold expression of IL-6 and IL-1β in a miscarriage toxoplasmosis women comparing with negative and positive control groups

Cytokine	Group	IL6-C_t value	GAPDH-Ct value	ΔC_t	$2^{-\Delta Ct}$	Fold expression
IL-6	Healthy control	25.3 ± 0.05	24.0 ± 0.06	1.00 ± 0.6	0.39 ± 0.60	0.98 ± 0.08
	Miscarriage (+ve)	23.47 ± 0.20	24.18 ± 0.08	0.71 ± 0.007	1.63 ± 0.05	2.11 ± 0.04
IL-1β	Healthy control	25.00 ± 0.34	24 ± 0.08	1.00 ± 0.37	0.02 ± 0.50	0.90 ± 0.13
	Miscarriage (+ve)	25.65 ± 0.34	24.18 ± 0.60	1.47 ± 0.05	0.36 ± 0.5	9.50 ± 0.11

C_t: threshold cycle; *: significant (P≤0.05); **: highly significant (P<0.01)

mediates their recruitment and activation of caspase-1, there by regulating caspase-1mediated maturation of IL-1β [19].

On the other hand, results indicated in Table 3 showed that there is an over expression in IL-6 gene in miscarriage toxoplasmosis women compared with the level of expression in healthy pregnant women as a control. Results also showed that highly significant (p≤ 0.01) transcriptional levels of IL-6 mRNAs in uninfected miscarriage women compared with healthy control group. Up-regulation of IL-6 gene expression indicates that peripheral blood lymphocytes of miscarriage toxoplasmosis women secrete high level of IL-1β and IL-6 which explain the role of these cytokines in the pathogenicity of recurrent miscarriage. The presence of inflammatory status and the role of IL-6 in resistance to the infection with *Toxoplasma* and contributes to a transformation from acute to chronic inflammation [20]. This deals with [21] who mentioned that serum levels of IL-6 were higher in miscarriage toxoplasmosis women more than in control groups. These results are similar to the findings obtained by [22], who mentioned that increasing levels of IL-6 gene expression is due to a higher miscarriage rate in toxoplasmosis women. By inducing apoptosis, IL-6 can promote the survival of infected cells, and conclusively established that IL-6 is involved in the T cell differentiation control between two essential CD4+Populations of T cells: T (Treg) regulatory cells and T helper 17 (Th17) cells. In particular, IL-6 stimulates the differentiation of Th17 cell in combination with TGF-β by improving the expression of RORγt [23]. Many studies considered that serum cytokines level as some markers in women with recurrent pregnancy loss or miscarriage using MILLIPLEX analysis causing a high level of Il-6 gene expression in women with recurrent miscarriage [24]. Al-Jameil et al. [25] suggests that there is a common gene expression response to *T. gondii* infection in mice (Fig. 2).

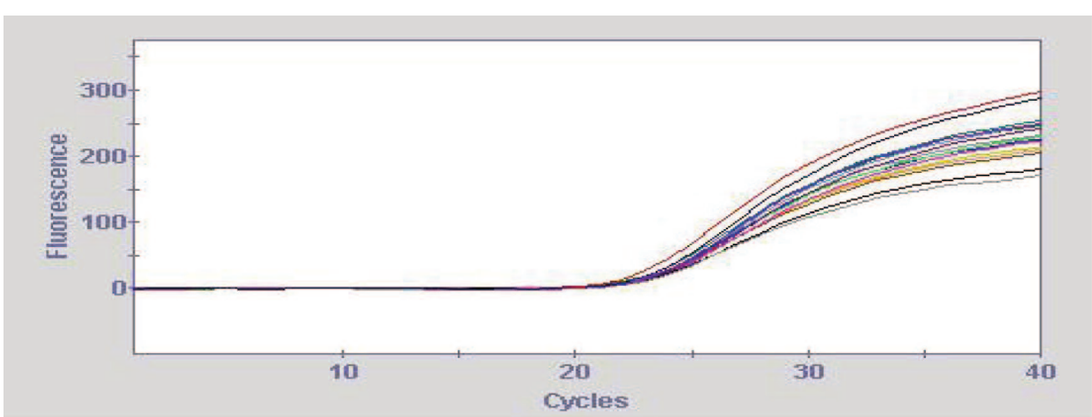

Figure 1. Melting curve analysis by RT-PCR for detection of *Toxoplasma gondii B1* gene in blood samples of aborted women. Blue, green plots: aborted women; red plot: healthy control women (DNA *Toxoplasma gondii*)

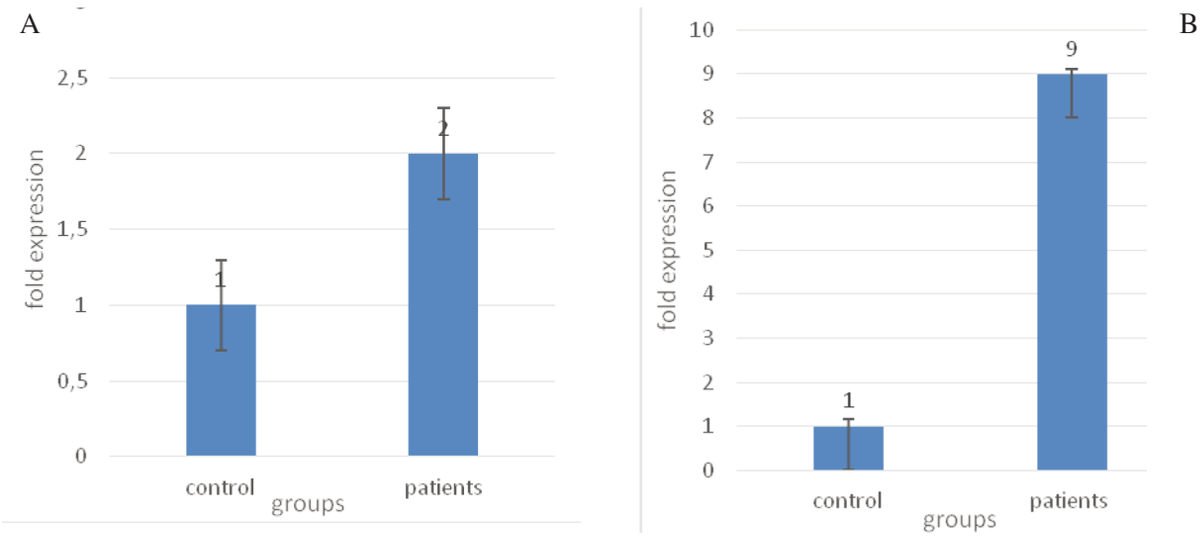

Figure 2. mRNA level of IL-6 and IL-1β analyzed using the 2-ΔΔCT method. A. show error bars of IL-6 in 3 groups. B. show error bars of IL-1β in 3 groups. Each error bar constructed using slandered deviation of fold expressions.

standard deviation error bars do not overlap, it's a clue that the difference may be significant.

This response is modified by parasite strain-specific factors that determine their distinct virulence phenotypes. He also noticed that induction of IL-1β needs active parasite attack of monocytes, meanwhile heat-killed or mycalolide B-treated parasites did not stimulate IL-1β production. Among the type I, II, and III strains of *T. gondii,* the type II strain induced substantially more IL-1β mRNA and protein release than did the type I and III strains. Our results showed that miscarriage Toxoplasmosis women has twice amount of IL-6 compared with healthy pregnant subjects, which seems to support the existence of an inflammatory condition. As a result, these cytokines increase lead to the activation of Natural Killer T-cytotoxic and phagocytic cells to be more effective in killing which can lead to pregnancy complication and fetus rejection due to large amount of apoptotic cells in the endometrium and placenta leading to miscarriage [25,26].

In conclusion, up-regulation of IL-6 and IL-1β expression may attributed to the infection with high-risk toxoplasmosis through its invasion proteins such as (Rop5 and Rop 16) and it could be a biomarker for detection of types I, II strains driven stronger immune responses lead to be a useful therapeutic target.

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