

The Expression of T-Helper Associated Transcription Factors and Cytokine Genes in Pre-Eclampsia

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ABSTRACT

Background: Pre-eclampsia (PE) is known as a main factor contributing to fetomaternal mortality, which might affect 2-8% of all pregnancies after the twentieth week of gestation. The balance of T helper subsets is essential to sustain a normal pregnancy and preventing fetomaternal complications. **Objective:** To investigate differences in the levels of transcription factors and cytokine gene expression of Th1/Th2/Th17/Treg subsets within decidual and chorionic layers of placentas from 15 PE-afflicted and 15 healthy Iranian women in their third trimester of pregnancy. **Methods:** Using Quantitative real-time PCR (Q-PCR), The expression of T-BET, GATA-3, ROR- γ t, FOXP3, and cytokines, including IL-1, IL-6, TNF- α , IFN- γ , IL-4, IL-31, IL-17, IL-23, TGF- β 1, TGF- β 2, TGF- β 3, and IL-35 in the placenta were compared at mRNA levels between groups. **Results:** FOXP3 and GATA-3 were significantly down-regulated, while T-BET was up-regulated in PE deciduae compared to the control group ($p < 0.0001$, $p < 0.02$, and $p < 0.01$, respectively). Concerning the chorionic samples, FOXP3 significantly decreased, while ROR- γ t increased in the PE placentas compared to the healthy ones ($p < 0.0006$ and $p < 0.02$, respectively). Besides, most inflammatory cytokines were up-regulated, while anti-inflammatory cytokines were down-regulated in the PE placentas. Additionally, TNF- α /IL-35, IFN- γ /IL-35, IL-6/IL-35, and IL-23/IL-35 ratios were significantly higher ($p < 0.01$) and IL-35/IL-17 ratio was significantly lower ($p < 0.05$) in the pre-eclamptic patients compared to the healthy controls. **Conclusion:** Our results shed more light on the contribution of Th1/Th2/Th17/Treg balance within placenta in the fate of a normal pregnancy. Moreover, regulatory T cells and IL-35 seem to play a central role in the regulation of all subsets.

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Keywords: Cytokines expression, IL-35, Pre-eclampsia, Transcription Factor

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INTRODUCTION

Preeclampsia (PE) is one of the most important factors in fetomaternal morbidity, which usually occurs after the twentieth week of gestation and affects 2-8% of all pregnancies worldwide (1). PE is diagnosed by new occurrence of hypertension (systolic BP >140 mm Hg and/or diastolic BP >90 mm Hg) along with proteinuria (at least >0.3 g or a urine dipstick reading of 1+ or greater in a 24-hour urine collection). Although the exact etiology of PE is still unknown, it is clear that the interplay between mother and placenta is impaired in PE and the only treatment for the disease is delivery of the placenta (2). Inappropriate activation of mother's immune system against fetal alloantigens and placental ischemia have also been shown in the previous studies (3,4). During pregnancy, a large population of immune cells exists in the maternal-fetal interface, which has diverse roles in different stages of pregnancy (5). Many chemokines and cytokines are also produced by cytotrophoblast and immune cells in the placental microenvironment where they communicate with placental alloantigens (6,7). T lymphocytes are one of the most important populations of the adaptive immune system in the maternal-fetal interface (8). Although T cells are really scarce in the early stages of gestation, they increase with the progression of pregnancy (9). T cells are divided into several subclasses based on their cytokine production (10), which might be associated with the success of pregnancy or incidence of pregnancy complications (11,12). Differentiation and activity of T cells are regulated by specific transcription factors, which promote differentiation of the related subclasses and prevent other subclasses. In this context, differentiation of CD4⁺ T cells into Th1 and Th2 can be regulated by T-bet and GATA-3, promoting production of pro-inflammatory or anti-inflammatory cytokines (13). Through generation of pro-inflammatory cytokines, such as IFN- γ , Th1 response participates in inflammatory responses. On the other hand, Th2 responses prevent induction of Th1 responses through production of anti-inflammatory cytokines, such as IL-4, IL-5, IL-10, and the newly described cytokine IL-31 (10,14,15). Although several previous studies have indicated that imbalance between Th1/Th2 responses is related to pathogenesis of PE (16,17), two more T cell subsets, named Th17 and regulatory T cells (Treg), have recently been added to this scenario.

Differentiation of Th17 cells is affected by the cytokine milieu of IL-1, IL-6, IL-21, and IL-23 and is controlled by the specific transcription factor, ROR- γ t (18,19). There is controversy about the role of TGF- β in Th17 differentiation and function (20,21). Th17 cells play a key role in inflammatory responses by production of pro-inflammatory cytokines, particularly IL-17 and IL-22 (22). Although inflammation caused by these cells is naturally important in defending the host against pathogens, they are related to pathogenesis of many inflammatory diseases (23). Moreover, since PE is a condition of systemic inflammatory response, Th17 cells may play a role in the induction of the disease (24,25). Treg cells differentiation is regulated by the transcription factor Foxp3 (26). In the absence of the inflammatory cytokines, TGF- β induces differentiation of T lymphocytes towards Treg cells (27). This type of CD4⁺ cells is one of the most important regulatory cues that attenuate inflammatory responses (28). Recently published studies have shown that during pregnancy, the frequency of regulatory T cells in the placenta significantly increases compared to the peripheral blood in the human pregnancies (29,30). It seems that Treg cells expand and prevent inflammatory responses through presentation of antigens by APCs in the fetal-maternal interface (31). IL-35 is a recently discovered immunosuppressive cytokine secreted by regulatory T

cells. IL-35 is a newly-described cytokine comprised of the IL-12 α chain (p35) and the Epstein-Barr virus-induced gene product (EBI-3) (32). Interestingly, IL-35 has been reported to be widely expressed on placental cells' surfaces (33). Considering the importance of Th1, Th2, Th17 and Treg subsets in pregnancy and pregnancy-related complication and controversial reports regarding their functions and related cytokines in PE, the present study aims to investigate differences in the expression of T helper subtype specific transcription factors (T-bet, GATA-3, ROR- γ t, and Foxp3 for Th1, Th2, Th17, and Treg, respectively) and their related cytokines (IL-1, IL-6, TNF- α , and IFN- γ for Th1, IL-4, IL-10, and IL-31 for Th2, IL-17 and IL-23 for Th17, and TGF- β 1, TGF- β 2, TGF- β 3, and IL-35 for Treg) at mRNA levels within placenta in a group of PE women at the time of cesarean section and healthy women.

MATERIALS AND METHODS

Study Population and Sampling. This case-control study was conducted on 30 pregnant women in the third trimester of pregnancy (gestational age ≥ 37 weeks, Table 1). Written informed consents for using the placentas approved by the local Ethics Committee of Shiraz University of Medical Sciences, Iran were obtained from all the participants. Among the participants, 15 women were diagnosed with PE and the remaining 15 were healthy without any history of hypertension, autoimmunity, infection, and cancer. Examination and selection of the participants were done by one gynecologist. The women with the history of recent infection, autoimmunity, cancer, and pregnancy-related complications were excluded from the study. The PE and healthy subjects were matched regarding age (mean age: 29.1 and 27.9 years, respectively) and gestational age (mean gestational age: 37.8 and 38.1 weeks, respectively). Diagnosis of PE was based on hypertension and proteinuria. Mild PE was defined as maternal blood pressure greater than 140/90 mmHg measured on two occasions with an at least 6-hr interval and proteinuria greater than 0.3 gr in a 24-hr urine specimen. The women with blood pressure above 160/110 mmHg and proteinuria of at least 5g were diagnosed as the severe form. Based on this classification, 5 and 10 women in the PE group were diagnosed with severe and mild types of the disease, respectively. The placental tissues were obtained by cesarean section and decidual and chorionic layers of the placentas were separated and processed separately. After washing with cold normal saline, the samples were stored in liquid nitrogen until extraction of total ribonucleic acid (RNA).

RNA Extraction. At this stage, extraction of total RNA from decidual and chorionic layers of the collected tissues was performed using a total RNA extraction kit (Pars Tous, Iran) based on the manufacturer's instructions. The concentration of the extracted RNA was determined using a Nano drop instrument (Thermo Scientific, USA). Immediately after the extraction, the RNAs were converted to cDNA. cDNA was synthesized using an easy TM cDNA synthesis kit (Pars Tous, Iran) based on the manufacturer's instructions. One microgram of total RNA was used for cDNA synthesis. Briefly, the mixture of 2 μ L oligo-dT, 2 μ L RNA, and 6 μ L DEPC water was heated at 65°C for 5 minutes and was immediately transferred on ice. After adding 10 μ L RT premix 2X, cDNA was made and stored in aliquots at -70°C. The quality of cDNA was examined by PCR method and agarose gel electrophoresis.

Quantitative Real-Time PCR. Quantitative real-time PCR (Q-PCR) was performed (Applied Bio systems step one Real-Time PCR system, USA) using the SYBR Green

method to amplify T-bet, GATA-3, ROR γ t, Foxp3, IL-1, IL-4, IL-6, TNF- α , IFN- γ , P-35, EBI-3, IL-31, TGF- β 1, TGF- β 2, TGF- β 3, IL-17, and IL-23. Primers were designed using Primer 3 online software v. 0.4.0. Briefly, 0.5 μ L of each primer (Table 2), 2 μ L of target cDNA (selected after checking and normalization of Ct values), 10 μ L of SYBR Green dye, and 0.4 μ L of ROX dye were used for amplification.

Table 1. Baseline characteristics of all the women in the study.

Parameters	Control (N=15)	Preeclampsia (N=15)	P value
mean age (years)	29.07 \pm 1.55	27.93 \pm 1.83	N.S
Mean gestational age (weeks)	38.07 \pm 0.22	37.80 \pm 0.49	N.S
Gravidity	2.4 \pm 0.19	1.93 \pm 0.3	N.S

Values are presented as Mean \pm SE. N.S= Not Significant, All P values were calculated using t-test.

Table 2. The primers used for RT-PCR analysis.

Gene	Forward primer	Reverse primer
IFN- γ	CTAATTATTCGGTAACTGACTTGA	ACAGTTCAGCCATCACTTGGGA
IL-1A	CCAACGGGAAGGTTCTGAAG	GGCGTCATTCAGGATGAATTC
IL-6	AAATTCGGTACATCCTCGAC	CCTCTTTGCTGCTTTTCACAC
TNF- α	GCCTGCTGCACTTTGGAGTG	TCGGGGTTCGAGAAGATGAT
TGF- β 1	AAATTGAGGGCTTTCGCCTTA	TGAACCCGTTGATGTCCACTT
TGF- β 2	CAGTGGGAAGACCCACATC	GCCGGTTGGTCTGTTGTGA
TGF- β 3	CGGAATGAGCAGAGGATCGA	GCCACCGATATAGCGCTGTT
IL-35 (EBI-3)	CCTTCACCACTCCCAAAAAC	GGCTTGATGATGTGCTCTG
IL-35 (P-35)	CCTTCACCACTCCCAAAAAC	TGTCTGGCCTTCTGGAGCAT
IL-23	CCAAGGACTCAGGGACAACCT	ATCAGGGAGCAGAGAAGGCT
IL-4	CCACGGACACAAGTGCATAT	GGCAGCAAAGATGTCTGTTACG
IL-31	GTGCTCTTTCTGTTCTGCTG	CATTGATGGGTGTCTGTTGG
Gata3	AGATGGCACGGGACACTACCT	GCCTTCGCTTGGGCTTAAT
Foxp3	CACCTGGAAGAAGCCATCC	CTCATCCACGGTGGTCCACACAG
ROR- γ t	CCCACAGATTTTGCAAGGGA	GCTGAGAAGGACAGGGAGCC
T-bet	AACACAGGAGCGCACTGGAT	TGGAGGGACTGGAGCACAAT
IL-17	GGAAGAAACAACGATGAC	GATTCCTGCCTTCACTAT
18srRNA	CTCAACACGGGAAACCTCAC	AAATCGCTCCACCAACTAAGAA

The final volume of each reaction was 20 μ L. Moreover, 18s rRNA was used as the housekeeping internal control gene. All the primer sets were used with reaction cycle conditions of 95°C for 15 s and 60°C for 34 s, except for T-bet primers that were used at 95°C for 30 s, 57°C for 20 s, and 72°C for 30 s. After the PCR reaction completed, the data were automatically analyzed by the instrument software and the copy number of the target genes and internal control genes in the samples were calculated. Comparative Ct and fold differentiation methods were used for quantification of target gene expression. Furthermore, 18s rRNA (control housekeeping gene) was used to normalize the Q-PCR. Melting curve analysis was also used for specificity of the amplified product.

2.4. Statistical Analysis. Using GraphPad Prism version 5.0 (GraphPad software Inc. San Diego, CA, USA), statistical analysis of the experimental data was performed and the results were expressed as mean \pm SEM. Differences between the study groups' mean values were assessed by Mann-Whitney test and $p < 0.05$ was considered as statistically significant. Δ CT values were used for comparison of the cases and controls regarding gene expression and $2^{-\Delta\Delta C_t}$ was used for relative gene expression analysis. Furthermore, the associations between the variables were analyzed by Spearman correlation coefficient using the SPSS statistical software, version 16 (SPSS Inc, Chicago, IL, USA).

RESULTS

The present study investigated and compared the level of Th1/Th2/Th17/Treg transcription factors (T-bet, GATA-3, ROR- γ t, and Foxp3, respectively) and their related cytokines at mRNA level in the decidual and chorionic layers of the placentas of the PE patients and healthy pregnant women.

Expression of Th1/Th2/Th17/Treg Transcription Factors in the Decidual Layer of the Placenta. According to Figure 1, maximum change in the expression levels of the studied transcription factors within the decidual layer was related to Foxp3. The expression of Foxp3 and GATA-3 transcription factors was significantly down-regulated within the decidual samples from the PE patients ($p < 0.0001$ and $p < 0.02$, respectively), while the expression of T-bet transcription factor was significantly up-regulated compared to the normal placental tissues ($p < 0.01$). However, no significant difference was found between the cases and controls regarding the expression of ROR- γ t in the decidual layer of the placenta.

Expression of Th1/Th2/Th17/Treg Transcription Factors in the Chorionic Layer of the Placenta. Similar to decidua, Foxp3 showed the maximum changes within the chorionic layer of the PE patients compared to the healthy women and was significantly decreased in the PE group ($p < 0.0006$, Figure 2). On the contrary, no significant difference was observed between the cases and controls concerning the expression of GATA-3 (Figure 2). Although the expression of T-bet within the chorionic layer was increased in the PE patients, the difference between the cases and controls was not statistically significant ($p < 0.06$, Figure 2). Finally, unlike decidua, the expression of ROR- γ t was significantly increased within the chorionic samples from the PE cases compared to the healthy ones ($p < 0.02$, Figure 2).

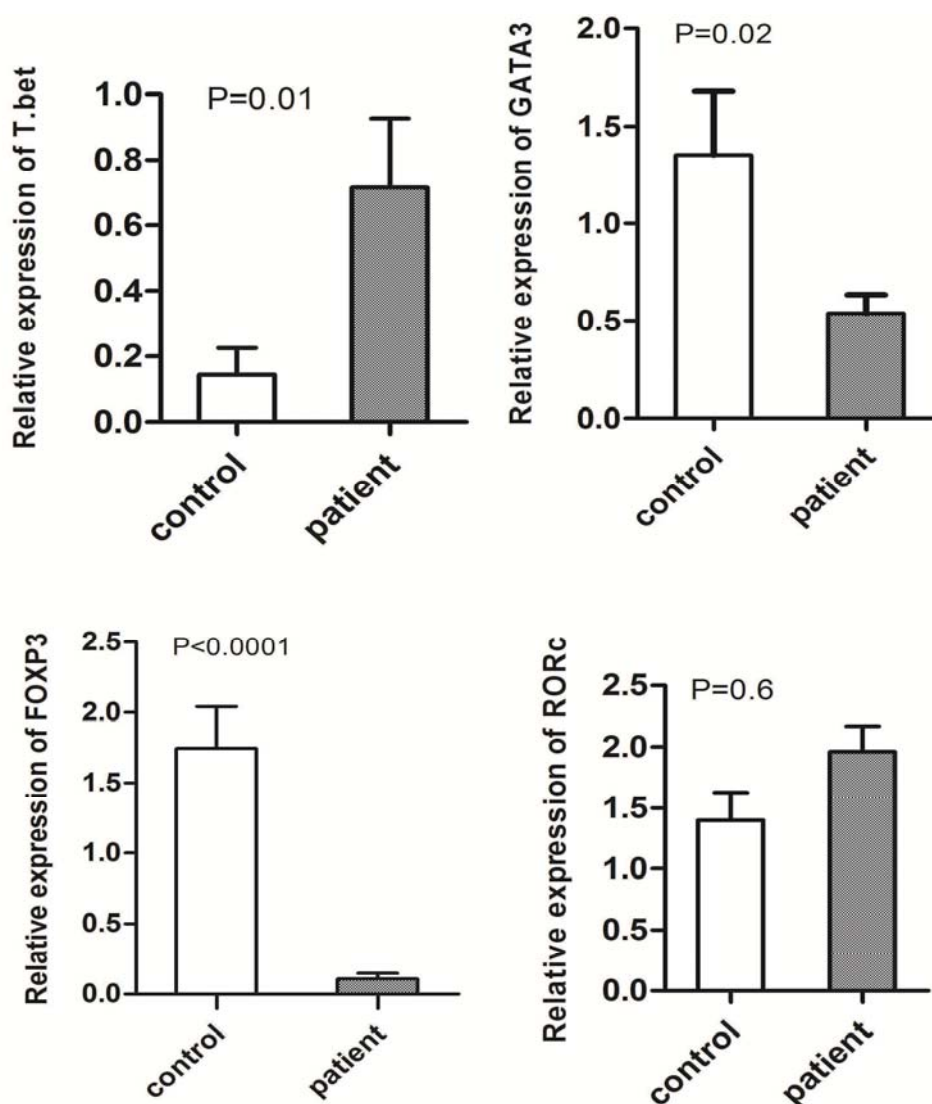


Figure 1. Comparison of the relative expression levels of T-bet, GATA-3, Foxp3, and ROR- γ t transcription factors in the decidua layers of the placentas from the pre-eclamptic and healthy pregnant women.

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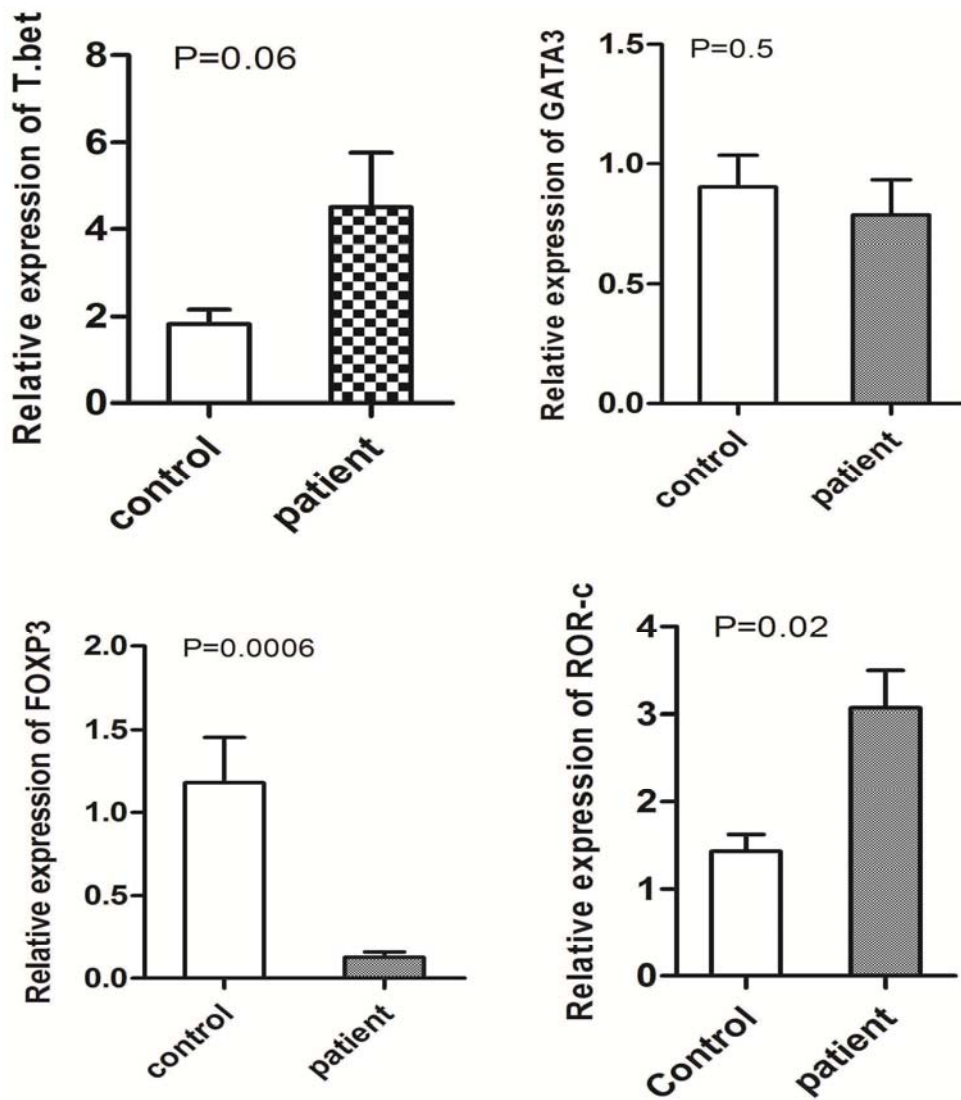


Figure 2. Comparison of the relative expression levels of T-bet, GATA-3, Foxp3, and ROR- γ t transcription factors in the chorionic layers of the placentas from the pre-eclamptic and healthy pregnant women.

Expression of the Studied Cytokines in the Decidual Layer of the Placenta.

Statistical analysis indicated that all the inflammatory cytokines, including IL-1, IL-6, TNF- α , and IFN- γ , were significantly up-regulated within the decidual layers of the PE women compared to the healthy ones (Figure 3, $p < 0.0001$ for all comparisons). Although the decidual layers from the PE women produced more IL-17 and IL-23 compared to the healthy women, these differences were not statistically significant (Figure 3, $p = 0.2$ and $p = 0.4$ respectively). In contrast, the expressions of all the studied anti-inflammatory cytokines, except for TGF- β 2, were significantly down-regulated in the PE compared to the healthy women (Figure 3). Considering IL-4 and IL-31, no expression was detected within the decidual layers from both PE and healthy women's placentas.

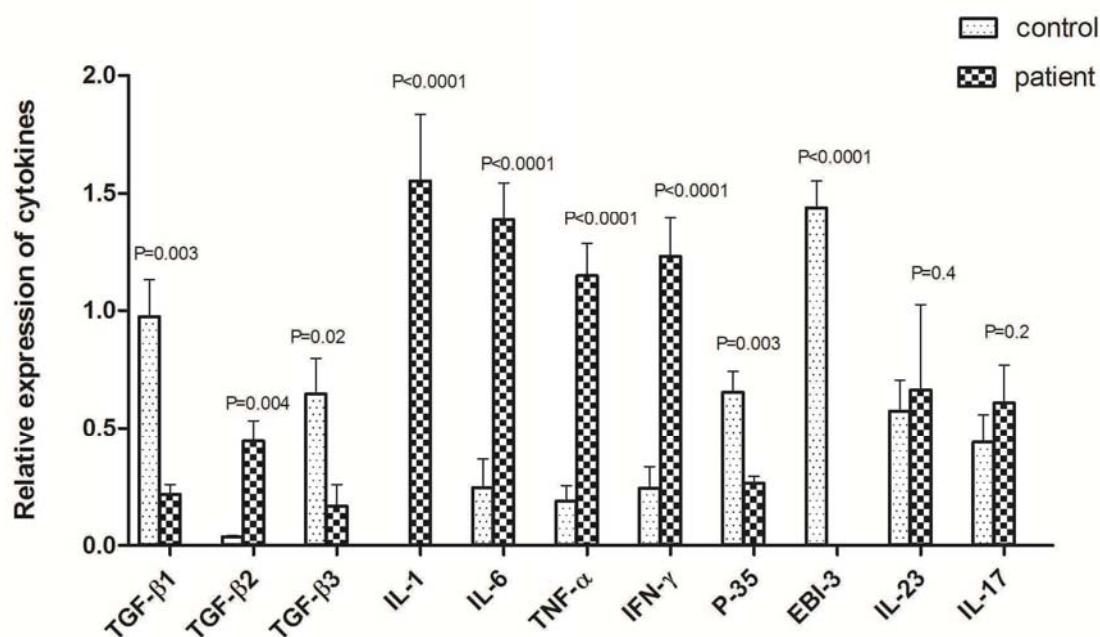


Figure 3. The expression of TGF-β1, TGF-β2, TGF-β3, IL-1, IL-6, TNF-α, IFN-γ, P-35, EBI-3, IL-23, and IL-17 mRNA in the decidua layers of the placentas from the pre-eclamptic and healthy pregnant women relative to 18s rRNA.

Expression of the Studied Cytokines in the Chorionic Layer of the Placenta. Within the chorionic layer, expression of all the inflammatory cytokines, except for IL-17, increased in the PE patients compared to the controls (Figure 4; $p < 0.0001$ for IL-1, IL-6, and TNF-α, $p < 0.0004$ for IFN-γ, and $p < 0.02$ for IL-23, respectively). On the other hand, expressions of TGF-β3 and EBI3 were significantly decreased in the PE patients compared to the controls ($p < 0.001$ and $p < 0.006$, respectively; Figure 4), while the two groups were similar with respect to the expression of TGF-β1, TGF-β2, and P35 (Figure 4). Moreover, no detectable expression of IL-4 and IL-31 in the chorionic layer was observed in the PE and healthy women.

Correlation between the Expressions of the T Cell Transcription Factors and their Related Cytokines. Spearman's correlation analysis indicated that the expression of most studied T cell transcription factors was correlated to their related cytokines. The expression of T-bet was correlated to TNF-α ($R = 0.77$, $p = 0.02$) and IL-6 ($R = 0.56$, $p = 0.04$) in chorion, while the expression of ROR-γt were correlated to IL-17 ($R = 0.65$, $p = 0.03$), and expression of Foxp3 was correlated to TGF-β1 ($R = 0.60$, $p = 0.02$) and TGF-β2 ($R = 0.52$, $p = 0.04$) in decidua layer. Furthermore, expression of GATA-3 was correlated to P35 subunit of IL-35 ($R = 0.60$, $p = 0.02$).

The Relationship between the Expressions of the Studied Cytokines. Spearman's correlation analysis showed a linear correlation between the expressions of most pro-inflammatory and anti-inflammatory cytokines. Accordingly, in the chorionic layer, the expression of TNF-α was correlated to IL-1 and IL-6 ($R = 0.89$, $p < 0.0001$ for both comparisons) and IFN-γ ($R = 0.52$, $p = 0.04$).

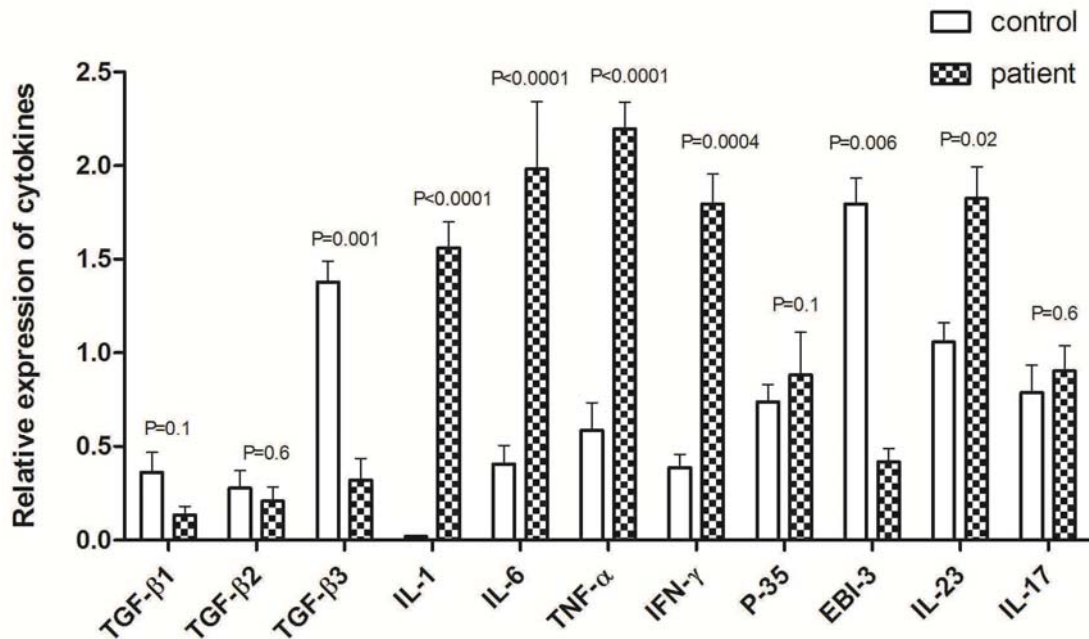


Figure 4. The expression of TGF-β1, TGF-β2, TGF-β3, IL-1, IL-6, TNF-α, IFN-γ, P-35, EBI-3, IL-23, and IL-17 mRNAs in the chorionic layers of the placentas from the pre-eclamptic and healthy pregnant women relative to 18s rRNA.

Furthermore, a strong correlation was also detected between the expressions of IL-1 and IL-6 ($R=0.98$, $p<0.0001$ for both comparisons) in this layer. Interestingly, the expression of IL-23 was correlated to that of TNF-α, IFN-γ, IL-1, IL-6, and IL-17 ($p<0.05$ for all comparison) in decidual layer. Moreover, the expression of TGF-β3 was correlated to P35 and EB13 subunits of IL-35 ($R=0.52$, $p=0.02$ and $R=0.56$, $p=0.04$, respectively). Interestingly, ratio analysis of the studied cytokines indicated that TNF-α/IL-35, IFN-γ/IL-35, IL-6/IL-35, and IL-23/IL-35 ratios were significantly higher ($p<0.01$), while IL-35/IL-17 ratio was significantly lower ($p<0.05$) in the pre-eclamptic patients compared to the healthy controls.

DISCUSSION

It is well known that a fine balance in immune responses is necessary for proper function of the immune system and maintenance of immune tolerance toward self and foreign antigens. Traditionally, Th1/Th2 paradigm is used for many years to illustrate the role of disturbance in immune balances in predisposing to pregnancy complications (34,35). After discovery of other T helper cell subsets, the paradigm is now extended to Th1/Th2/Th17/Treg balance (10,36). Considering the complexity of T helper subsets and their related cytokines in controlling the immune responses, to illustrate a comprehensive picture from what happens within placental tissue, the present study investigated T helper subsets and their related cytokines simultaneously in a group of

Iranian PE patients. In contrast to the previously published papers, this study evaluated T helper subsets and their related cytokines within and also in different parts of the placental tissues; including decidual and chorionic layer (25,30,31,35). Although most of the results obtained in our study are in line with the previous reports, many interesting findings are reported for the first time. In agreement with the recent studies (37,39,40), the results of the present study indicated that regulatory T cells played an important role in maintenance of a normal pregnancy and were down-regulated in the pre-eclamptic women. The expression of Treg transcription factor and most related cytokines was also significantly down-regulated within both sides of the placentas. In line with most reported data (17,41,42,43), the expression of Th1-related transcription factor and its related cytokines showed up-regulation in both chorionic and decidual sides of the placentas from the pre-eclamptic women. Consistent with the previously published studies (17,44,45), the current study results demonstrated a decrease in expression of Th2 transcription factors and their related cytokines and an increase in that of Th17 transcription factor and its related cytokines in the pre-eclamptic women (46,47). Interestingly, we did not detect IL-4 and IL-31 at mRNA expression. Considering this point that our cases and healthy women were all at the end of the pregnancy period, no detection of IL-4 and IL-31 is not surprising because at the end of the pregnancy Th2 responses are down-regulated. Moreover, our results suggested that IL-23 might play a more important role in maintenance of Th17 responses within the placental tissue.

The most interesting finding of the present study was the role of IL-35 in PE. IL-35 was discovered in 2007 and is composed of a specific EBI-3 and a non-specific P-35 subunit (32). By inducing a reduction in IL-17, IL-22, and Th17 transcription factor, the EBI-3 subunit seems to decrease the differentiation of Th17 cells (29). IL-35 is produced by iTreg cells and, by an autocrine positive effect, leads to proliferation of iTreg cells (48). Considering the expression of IL-35 by the placenta and its regulatory function, this cytokine might play a key role in maintenance of a normal pregnancy. The results of the present study supported this hypothesis and showed that both subunits of IL-35 (P35 and EBI3) were significantly decreased at mRNA level within the decidual layer of the placenta from the pre-eclamptic women compared to the healthy ones. Similar to the decidua, expression of the EBI-3 subunits was decreased in the chorionic layer from PE patients. However, no change was observed in the expression of P-35 subunit, which is probably due to the non-specificity of the P-35, a common subunit of IL-27 cytokine. In line with our results regarding the expression of IL-35, a recently published paper reported a significant decrease in IL-35 at mRNA level in peripheral blood samples from pre-eclamptic women (44). Ratio analysis of the studied cytokines also confirmed the important role of IL-35 in the regulation of all the subsets. According to the present study results, TNF- α /IL-35, IFN- γ /IL-35, IL-6/IL-35, and IL-23/IL-35 ratios were significantly higher, while IL-35/IL-17 ratio was significantly lower in the placental samples from the pre-eclamptic patients compared to the controls. Yet, this interesting finding need to be confirmed in future studies.

In conclusion, the results of the present study indicated that Th1/Th2/Th17/Treg balance within the placental tissue determined the fate of a normal pregnancy. Moreover, it seems that regulatory T cells play a central role in the regulation of all the subsets. The results also highlighted the role of a new regulatory cytokine, IL-35, in balancing the T helper responses in the placenta.

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