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Original Research Article

Levels of IL-17 and IL-35 in Chinese women as biomarkers of preeclampsia, and their pharmacological association with Traditional Chinese Medicine

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Abstract

Purpose: *To determine the serum levels of IL-17 and IL-35 in preeclamptic Chinese women, and their pharmacological association with Traditional Chinese Medicine (TCM) compounds.*
Methods: *In this study, 60 preeclamptic and 60 healthy pregnant Chinese women comprised the study and control groups, respectively. The serum levels of IL-17 and IL-35 were determined using the ELISA technique. Moreover, the anti-inflammatory effects of 5 TCM compounds were determined with respect to their inhibitory potential against IL-10 and IL-35 in RAW 264.7 cells.*
Results: *There were statistically significant differences in mean serum levels of IL-17 and IL-35 between preeclamptic patients and control patients ($p < 0.05$). Furthermore, the serum levels of the two cytokines were significantly higher in patients with severe preeclampsia than in patients with mild preeclampsia ($p < 0.05$). Significant positive correlations were observed between systolic blood pressure of patients and levels of IL-17 and IL-35. The 5 TCM compounds showed good inhibitory potential against IL-17 and IL-35, when compared to the reference drug and positive control ($p < 0.05$).*
Conclusion: *IL-17 and IL-35 are useful inflammatory biomarkers for preeclampsia in Chinese women, and can potentially be used to assess the severity of the disease. In addition, TCM compounds which inhibit IL-17 and IL-35 may be useful therapeutic agents for controlling preeclampsia.*

Keywords: *Preeclampsia, IL-17, IL-35, Inflammatory biomarker, Traditional Chinese Medicine*

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INTRODUCTION

Preeclampsia is a major complication of pregnancy in women, and it is defined as hypertension and proteinuria following 20 weeks of gestation. It is among the major reasons of pregnancy-related deaths globally, and it is also associated with prenatal mortality, preterm birth and intrauterine growth irregularities [1]. The exact pathogenesis and aetiology of

preeclampsia are not clearly understood, although it has been recognized that placental insufficiency due to impaired implantation is a key factor in initiation of preeclamptic condition [2]. However, pronounced inflammatory response due to activation of innate and acquired immunities are associated with pathogenesis of disease.

The lymphocytic cells known to play important antagonistic roles are T-regulatory (Treg) cells and T-helper-17 (Th17) cells [3]. During the course of pregnancy, Treg cells inhibit the immunological response of mother against fetal tissue. Thus, a reduction in the number of Treg cells is often an indication of improper implantation. On the other hand, Th17 cells induce inflammatory and autoimmune responses in humans. It has been revealed that many obstetric complications are associated with an increase in number of Th17 cells along with reduced number of Tregs [4].

Therefore, there is need to maintain a balance between Th17 cells and Treg cells so as to provide a safe environment for the fetus. However, in preeclamptic pregnancies, an increase in the number of Th17 cells and a decrease in the number of Th17 cells have been reported [5,6]. This leads to dysregulation of several cytokines with inflammatory and anti-inflammatory effects on feto-maternal tissue, with circulating levels in maternal serum. These cytokines include IL-17 and IL-35 which are known to cause antagonist effects [7,8]. Interleukin-17 (IL-17) is secreted by Th17 cells, and it is known to elicit a variety of inflammatory responses which include recruitment and activation of neutrophils [2, 9]. The IL-17 family comprises 6 cytokines, namely IL-17A - IL17F, with IL-17A as the most important and well-studied cytokine. Physiologically normal serum levels of IL-17 are observed in normal pregnancy; these appear to be critical for maintaining immunological balance in feto-maternal interface [10,11]. Increases in levels of IL-17 in feto-maternal tissues as well as in circulating maternal serum have been reported in recent studies [12,13]. However, the actual role of IL-17, and its correlation with preeclamptic symptoms still needs to be elucidated [12,13].

Interleukin-35 (IL-35) is considered a cytokine from IL-12 family, and it is secreted from Treg cells. A recent study has demonstrated that IL-35 is also consistently produced by trophoblast cells during normal gestation, and it is a critical cytokine for maintaining feto-maternal tolerance [14,15]. Some recent reports showed elevated serum levels of IL-35 in women with preeclampsia [16-18]. Presently, no investigator has studied the level of these cytokines in Chinese preeclamptic population. This was the aim of the present study. The circulating serum levels of IL-17 and IL-35 in a group of Chinese women with preeclampsia were determined, as well as the correlation of these cytokines with the systolic blood pressure of the subjects.

METHODS

Ethical approvals

The study was approved by the Institutional Medical Ethics Committee of Zhuhai People's Hospital, China (approval ref no. ZH/LS-19-32145). Written informed consent was provided by all participants. The study protocol followed was in accordance with the Declaration of Helsinki 1964 and its later amendments [19].

Study design and subjects

The study was performed on 70 women diagnosed with preeclampsia (PE group) and 70 healthy pregnant women (control group). The preeclamptic women were recruited from patients attending the antenatal outpatient ward. The inclusion criteria for preeclamptic women were: blood pressure ≥ 140/90 mmHg, and 24 h urine protein ≥ 0.3 g at gestational age between 28 to 34 weeks; and preeclampsia diagnosed as defined by the American College of Obstetricians and Gynaecologists (ACOG). The PE groups were further categorized into sub-groups of mild and severe preeclampsia. There were 27 patients with blood pressure ≥160/110 mmHg and 24 h urine protein ≥5.0 g, and they were considered severe; whereas, the other 33 patients were considered mild. Patients with coexisting inflammatory and/or autoimmune disorders, renal dysfunction, angiopathy, diabetes mellitus, infectious disease and fetal congenital anomalies were excluded. The inclusion criteria for the control group were identical to those of the study group, except for symptoms related to preeclampsia. Blood pressure was recorded at the time of admission for the groups. Moreover, blood pressure was determined for the study group subjects every 4 h starting at a particular time every morning. The patients were provided a management regimen of antihypertensive drugs, and they were closely monitored for 2-weeks.

Cytokine assay

The serum levels of IL-17A and IL-35 were measured after collecting 5-ml blood samples

from participants under aseptic conditions in the Immunology Laboratory. Serum samples were isolated from the blood samples using cold centrifugation, and the sera were kept frozen at -20 °C prior to use so as to prevent loss of bioactive cytokines. Invitrogen™ IL-17A (homodimer) Human Uncoated ELISA Kit (Thermo Fisher Scientific Inc., USA; 50-112-8758) was used to assay IL-17A as per manufacturer's instructions. The absorbance of all samples was read at a wavelength of 450 nm. The concentrations of IL-17 were calculated as pg/ml, and the sensitivity of the IL-17A kit was 0.5 pg/ml. For IL-35 assay, Human IL-35 (Interleukin 35) ELISA Kit (Elabscience Inc., E-EL-H2443, USA) was used according to manufacturer's instructions. The optical density of each sample was measured spectrophotometrically at a wavelength of 450 nm. The sensitivity of the IL-35 kit was 9.38 pg/ml.

Determination of anti-inflammatory effect of TCM compounds against IL-17 and IL-35

Five TCMs were screened to determine the anti-inflammatory effects of Traditional Chinese Medicine (TCM) used for treating preeclampsia. Five TCM compounds viz. salvianolic acid, pachymc acid, riligustilide, sodium ferulate and leonurine present in *Salvia miltiorrhiza* (Dan Shen), Poriaecocos (Fu Ling), *Ligusticum striatum* (Chuan Xiong), *Angelica sinensis* (Dang Gui) and *Leonurus japonicas* (Mu Cao) were obtained from Sigma–Aldrich (Darmstadt, Germany). They were screened for anti-inflammatory effects against IL-17 and IL-35. For this purpose, LPS-stimulated macrophages from blood RAW 264.7 Cell Line Murine (910627021VL, Merck KGaA, Darmstadt, Germany) were seeded in a 96-well plate and made adherent by incubating the cells for 18-24 h at 34 °C. The RAW 264.7cells were treated with 25 µM of each of the 5 TCM compounds, along with DMSO and indometacin (positive control) for 32 h. After 48 h, the incubation media of all the TCM compounds were centrifuged at 8000 rpm for 60 min. The cell supernatant was assayed for levels of IL-17 and IL-35 using standard ELISA kit (Millipore, USA) in line with manufacturer's protocols. The levels of IL-17 and IL-35 in the RAW 264.7 cells were measured and read in Genios Pro microplate reader (Tecan, Crailsheim, Germany).

Statistical analysis

The GraphPad Prism statistical software version 7.0 (GraphPad Software Inc., USA) was used for data analysis in the present study. One-way ANOVA, Student's *t*-test and Tukey's Post Hoc tests were utilized for the analysis. Fisher's exact test or Chi-square test was employed for analysis of categorical variables. Values of $p < 0.05$ were considered indicative of statistically significant difference.

RESULTS

The clinical characteristics of the studied groups are shown in Table 1. There were no statistically significant differences in age and gestational status between the healthy subjects (control group) and PE group ($p > 0.05$). Furthermore, there was no significant difference in ages and gestational status between patients having mild preeclampsia and those with severe preeclampsia ($p > 0.05$). The patients were classified into sub-groups of 33 mildly and 27 severely preeclamptic subjects (denoted as MPE and SPE groups, respectively). Diastolic and systolic blood pressures differed significantly between the control group and PE group, as well as between mild and severe preeclampsia sub-groups.

Results of quantitative assessments of levels IL-17 and IL-35 in the studied groups are presented in Table 2. The mean serum level of IL-17 was significantly higher in the preeclampsia group (8.81±2.12 pg/mL) and mild preeclampsia group (7.42±1.23 pg/mL) than in healthy control group (4.33±0.68 pg/mL; $p < 0.001$). Expectedly, the mean serum level of IL-17 was significantly higher in patients with severe preeclampsia (10.51±1.69 pg/mL) than in subjects in the healthy control group (4.33±0.68 pg/mL; $p < 0.001$).

Moreover, it was observed that the mean serum level of IL-17 was significantly higher in patients with severe preeclampsia (10.51±1.69 pg/mL) than in patients with mild preeclampsia (7.42 ± 1.23 pg/mL; $p < 0.001$).

The mean serum level of IL-35 in the preeclampsia group was 730.02 ± 195.15 pg/mL, whereas for the healthy control group, the mean serum level of IL-35 was 495.25 ± 62.49 pg/ml was significantly lower. Moreover, the mean serum level of IL-35 was significantly higher in patients with mild preeclampsia (586.12 ± 96.27 pg/mL) than in the healthy control group (495.25 ± 62.49 pg/mL; $p < 0.001$). In contrast, the mean serum level of IL-35 was significantly higher in patients with severe preeclampsia (905.89±128.88 pg/mL) than in healthy control group (495.25 ± 62.49; $p < 0.001$). Patients with severe preeclampsia had significantly higher IL-35 level (912.63 ± 114.43

pg/mL) than patients with mild preeclampsia (586.12±96.27 pg/mL; $p < 0.001$).
In contrast, the mean serum level of IL-35 was significantly higher in patients with severe preeclampsia (905.89±128.88 pg/mL) than in healthy control group (495.25 ± 62.49; $p < 0.001$). Patients with severe preeclampsia had significantly higher IL-35 level (912.63 ± 114.43 pg/mL) than patients with mild preeclampsia (586.12±96.27 pg/mL; $p < 0.001$).

Correlation analysis indicated that mean serum levels of both cytokines were higher in subjects with higher systolic blood pressure (Figure 1). A strongly positive correlation was observed between serum IL-17 levels and systolic blood pressure (R^2=0.818), whereas moderately positive correlation was found between serum IL-35 levels and systolic blood pressure (R^2=0.623). Table 3 shows the results of ELSIA assay for the inhibition of IL-17 and IL-35 from the cell supernatant assay of RAW 264.7 cells by 25 µM of each of the TCM compounds. In terms of inhibitory concentrations against IL-17, the results showed that salvianolic acid (11.27 ± 0.15 µM), riligustilide (12.24 ± 0.41 µM) and pachymic acid (13.76 ± 0.51 µM) were more effective than indometacin (reference drug, 14.34 ± 0.23 µM) and the positive control (16.38 ± 0.13 µM). Moreover, pachymic acid (10.21 ± 0.71 µM), salvianolic acid (12.51 ± 0.44 µM), leonurine (12.52 ± 0.64 µM) and riligustilide (14.82 ± 0.13) showed higher inhibitory potential against IL-35, when compared to indometacin (reference drug 15.65 ± 0.31) and the positive control (Kit, 17.31 ± 0.22). Sodium ferulate had poor inhibitory potential against IL-17 (16.73 ± 0.41 µM) and IL-35 (17.89 ± 0.21 µM), when compared to the reference drug and the positive control.

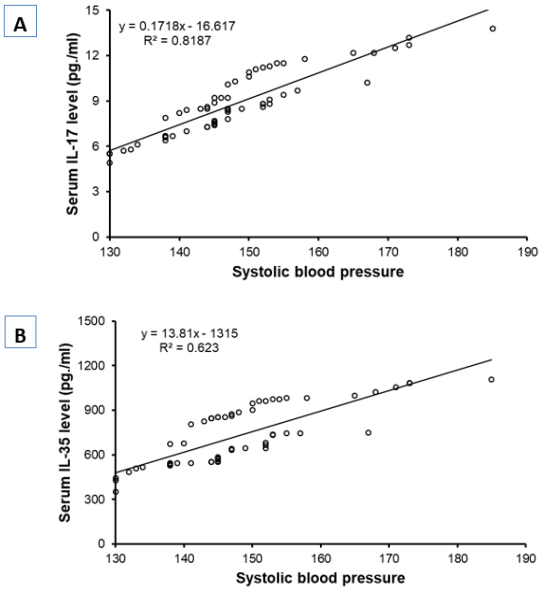

Figure 1: Correlation between serum levels of IL-17 and IL-35 with systolic blood pressure of the subjects

Table 1: Clinical characteristics of subjects in control and PE groups ((mean ± SD)

Parameter	Control group (*n*=60)	PE group (*n*=60)	*P*	MPE group (*n*=33)	SPE group (*n*=27)	*P*
Subject age (years)	28.52±3.95	29.62±3.454	0.109	29.64±4.05	29.59±2.86	0.963
Gestational age (weeks)	30.11±1.86	30.75±1.97	0.072	30.45±1.97	31.11±1.973	0.128
Diastolic BP (mmHg)	81.27±3.81	91.65±5.74	<0.001	89.33±5.16	94.48±56.71	0.001
Systolic BP (mmHg)	121.53±4.50	148.02±11.157	<0.001	144.12±8.62	152.78±12.15	0.002

Table 2: Comparison of serum levels of IL-35 and IL-17 (pg/ml) in control and preeclampsia groups

Cytokine	Control group (*n*=60)	PE group (*n*=60)	MPE group (*n*=33)	SPE group (*n*=27)
IL-35	495.25±62.49	730.02±195.15	586.12±96.27	905.89±128.88
IL-17	4.33±0.68	8.81±2.12	7.42±1.23	10.51±1.69

Table 3: Inhibitory potential of TCM compounds and standards against IL-17 and IL-35

S/no.	Plant source	Chinese name	Compound	IL-17 inhibition (µM)	IL-35 inhibition (µM)
1	Positive control (Kit)		-	16.38 ± 0.13	17.31 ± 0.22
2	Negative control (Kit)		-	53.16 ± 0.38	59.82 ± 0.21
3	DMSO		DMSO	295 ± 0.67	327± 0.56
4	Indometacin (Reference)		Indometacin	14.34 ± 0.23	15.65 ± 0.31
5	*Salvia miltiorrhiza*	Dan Shen	Salvianolic acid	11.27 ± 0.15	12.51 ± 0.44
6	*Poriaecocos*	Fu Ling	Pachymic acid	13.76 ± 0.51	10.21 ± 0.71
7	*Ligusticum striatum*	ChuanXiong	Riligustilide	12.24 ± 0.41	14.82 ± 0.13
8	*Angelica sinensis*	Dang Gui	Sodium ferulate	16.73 ± 0.41	15.19 ± 0.21
9	*Leonurus japonicas*	Mu Cao	Leonurine	14.11 ± 0.26	12.52 ± 0.64

DISCUSSION

The significance of immunological and inflammatory response at the placental site has been emphasized in recent studies related to the development of pathogenicity. In fact, several unfavorable sequelae in the third trimester of pregnancy are linked to preeclampsia. The cytokines IL-17 and IL-35 have been increasingly recognized as biomarkers of preeclampsia, especially owing to their association with changes in blood pressure. While few recent reports are available on up-regulated serum levels of IL-17 and IL-35 in preeclamptic women, the present study is perhaps the first to demonstrate such effect in a Chinese population. Chinese preeclamptic women were enrolled in late gestation (approximately 28 to 34 weeks), and comparison of levels of serum cytokines was performed, with a control group comprising healthy Chinese women of comparable ages and gestational status. This study showed that the levels of IL-17 and IL-35 were significantly elevated in the serum samples from preeclamptic women than in serum samples from healthy females. The increases in levels of IL-17 and IL-35 were related to disease severity, since the women with severe preeclampsia showed significantly higher serum levels of these cytokines than those with mild preeclampsia. A study of the limited reports concerning this phenomenon amongst different patient populations revealed both conflicting as well as supporting information.

For example, contrary to the findings in this study, Ozkan *et al* reported reductions in serum levels of IL-17 cytokine in a study carried out in 40 pregnant females with preeclampsia [17]. Moreover, Cao *et al*. investigated the mRNA and protein expressions of the two cytokines in Chinese women with preeclampsia, and reported marked down-regulation of IL-17 in women with preeclampsia [16]. However, Batebi *et al* found no significant difference in mean serum levels of IL-17 between preeclampsia group and control group (2.86 *vs.* 2.79 pg/ml) [20]. On the other hand, Martínez-García *et al* observed an increase in the circulating level of IL-17 during the third trimester *vis-à-vis* the first trimester (37.28 *vs.* 14.61 pg/mL), which is clearly consistent with the results of the present study, although it focused on late pregnancy only [21].

Similarly, in line with the present study, El Shahaway *et al* have reported a statistically significant increase in mean serum level of IL-17 in preeclampsia group, relative to control group (18.5 *vs.* 4.38 pg/mL) [4]. Similarly, studies with conflicting results have been observed concerning serum IL-35 levels in preeclampsia. The previously mentioned studies of Ozkan *et al* and Cao *et al* reported decreases in mean serum levels of IL-35 in preeclamptic females [16,17]. These investigators reported serum IL-35 values between 6.65 and 17 pg/ml, which appear to be significantly lower than physiological IL-35 levels (>120 pg/ml). Moreover, the findings of the present study support that of Batebi *et al* who reported significantly increased level of IL-35 in preeclampsia group (729 *vs.* 483.9 pg/ml) [19]. Patients with elevated systolic blood pressure generally showed more pronounced increases in levels of the two cytokines.

The present study shows a clear association between levels of the two cytokines and systolic blood pressure of the subjects. To the best of our knowledge, there is no study the correlation between blood pressure and these cytokines. The ELISA assay for inhibition of IL-17 and IL-35 revealed the anti-inflammatory potential of the TCM compounds, especially salvianolic acid, riligustilide and pachymic acid which had better inhibitory potential than indometacin (reference drug) and the positive control (Kit). In fact, IL-10 and IL-35 have been reported to play vital roles in immunological and inflammatory responses related to the pathogenic development of preeclampsia at the placental site [22]. Therefore, the inhibitory functions of IL-17 and IL-35 by TCM compounds such as salvianolic acid, riligustilide and pachymic acid, may provide new strategies for treating preeclampsia.

The present findings warrant more specific and comprehensive investigations on the cytokine network in preeclamptic patients. The authors would like to highlight few considerations which should be attached to the present study as well as other studies on this subject. Firstly, the enrolled subjects were under direct supervision of physicians who administered antihypertensive and/or other medications as required. Moreover, due to compliance issues, the current study focused only on the serum levels of the cytokines, whereas critical events related to these immune system modulators also take place at feto-maternal interface.

Lastly, it is emphasized that the immune responses in pregnant females are elicited in a time-dependent manner: the present study was limited to later stages of pregnancy. All subjects in the present study were expecting labor and were constantly monitored by physicians. Detailed analysis of the present results which

included measurements on a wider array of inflammatory biomarkers at different stages of pregnancy, revealed a more accurate and precise information on the association of these cytokines with pathophysiology of preeclampsia. The TCM compounds viz. salvianolic acid, riligustilide and pachymic acid which are present in *Salvia miltiorrhiza* (Dan Shen), *Ligusticum striatum* (Chuan Xiong) and *Poriaecocos* (Fu Ling), respectively, proved to be important leads by inhibiting both IL-17 and IL-35.

CONCLUSION

These results indicate that IL-17 and IL-35 are useful inflammatory biomarkers for preeclampsia in Chinese women. Thus, they can potentially be used to assess the severity of preeclampsia. Moreover, TCM compounds such salvianolic acid, riligustilide and pachymic acid, which inhibited IL-17 and IL-35, may be potent therapeutic agents for preeclampsia.

DECLARATIONS

Acknowledgement

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Conflict of interest

The authors report no conflict of interest in the present work.

Contribution of authors

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Lina Wang, Yu Liu, Shude Liu, Caiqin Ling, KuiLi, Qizhi Xiao and Xiong Liang designed all the experiments and revised the paper. Ina Wang, Yu Liu, Shude Liu, Caiqin Ling and Kui Li performed the experiments. Lina Wang, Yu Liu, Shude Liu, Caiqin Ling, KuiLi, Qizhi Xiao and Xiong Liang wrote the manuscript.

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