# Orignal Article:
# Comparison of TGF-β and Nitric Oxide Production by Adipose-Derived Mesenchymal Stem Cells Between Healthy Pregnant and Preeclamptic Women

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## ABSTRACT

**Background:** Preeclampsia is one of the most common complications of pregnancy that occurs after the 20th weeks of pregnancy. The pathophysiology of this disease is not exactly known. Transforming Growth Factor-Beta (TGF-β) and Nitric Oxide (NO) are the key regulatory factors secreted by Mesenchymal Stem Cells (MSCs). The aim of the present study was to evaluate the TGF-β and NO secretion by adipose-derived MSCs in normal and preeclamptic pregnant women.

**Materials and Methods:** The adipose tissues were collected from 10 preeclamptic patients and 10 age-matched normotensive controls at the time of cesarean section delivery. After isolation and expansion of MSCs, their capability of differentiation and immunophenotyping characteristics were assessed. Next, the release of TGF-ß was evaluated making use of ELISA sandwich method and Griess method was used to measure the level of NO.

**Results:** Adipose derived MSCs in both groups were differentiated into osteocytes and adipocytes. The expression of CD90, CD73, CD44, and CD105 markers and lack of expression of CD-14, CD34, CD45, and HLA-DR markers in cells isolated from adipose in both groups was observed using flow cytometric analysis. The levels of TGF-ß secretion in preeclamptic women were significantly higher than those in control group, but the mean level of NO secreted by adipose derived MSCs did not significantly change in the two groups.

**Conclusion:** It can be concluded that significant increase in the secretion of TGF-β owing to MSCs in preeclamptic participants shows the importance of these cells in controlling immunological balance in these patients. Therefore, MSCs-based therapy seems to regulate TH1/Th2 balance in preeclampsia.

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## Introduction

Hypertensive disorders complicate 5-10% of pregnancies and, together with hemorrhage and infection, are known to be the three leading causes of maternal mortality around the world [1]. Preeclampsia (PE) syndrome is the most complicated hypertensive pregnancy disorder [2]. PE syndrome, which is identified in 3.9% of all pregnancies, occurs after the 20$^{th}$ weeks of pregnancy in women with normal blood pressure and is characterized by hypertension and proteinuria [3]. PE can be the prelude of eclampsia, placental abruption, and Disseminated Intravascular Coagulation in the mother and preterm delivery, intrauterine growth restriction, and neurological disorders in the fetus [1, 4, 5]. Due to a lack of understanding of the etiology of preeclampsia, there is no way to predict, prevent, and treat this disorder and, therefore, the only way to prevent maternal morbidity and mortality is termination of pregnancy regardless of gestational age [6].

In preeclampsia, incomplete and shallow invasion of the cytotrophoblast to uterine spiral arteries leads to lack of adequate dilation of the arteries, reduced fetoplacental transfusion, placental ischemia, and hypoxia in spite of progression of pregnancy [4, 7]. Although most studies suggest that trophoblast cells are responsible for changes in preeclampsia, since human placenta is an organ that contains heterogeneous cell populations like endothelial cells, progenitor cells, myofibroblasts, and Mesenchymal Stem Cells (MSCs), it seems that all these Cells can play a role in maintaining the placenta function. A recent study showed that MSCs isolated from placenta can affect vasculogenesis through secretion of paracrine factors and are involved in protection against ischemic damage via anti-inflammatory and anti-fibrotic activities [8, 9]. Therefore, it is likely that impaired anti-inflammatory and pro-angiogenic activity of MSCs are involved in the pathogenesis of preeclampsia.

The most important source of stem cells is the bone marrow, but recently these cells have also been isolated from secondary sources, such as adipose tissue [10], placenta [11], umbilical cord [12], and peripheral blood [13]. Several Studies have shown that MSCs isolated from adipose tissue are similar to those isolated from bone marrow and placenta in morphology and expression of surface markers, cell behavior in cell culture, and power of differentiation to bone, cartilage, and fat cells [14, 15]. The advantages of separating the cells from adipose tissue include easy isolation, accessibility, and high frequency of these cells in this source [10].

Unlike embryonic stem cells, MSCs are highly immunosuppressive. Although the interaction between MSCs and target cells is important for inducing immune suppressive activity of these cells, the bulk of this activity is performed by secreting soluble factors, such as IL-10, TGF-β, Nitric Oxide (NO), Indoleamine 2,3-Dioxygenase, and Prostaglandin E2, which are induced or increased following the interaction with target cells [16-18]. It is worth noting that the immunosuppressive activity induced by MSCs is a complex process and inhibition of only one of the soluble factors does not inhibit this activity [19].

Immunosuppressive ability of MSCs is not intrinsic and is induced by inflammatory cytokines, such as IFN-γ, IL-1α, IL-1β, and TNF-α. In normal pregnancy, immune responses shift from Th1 to Th2 responses to maintain the favorable conditions, but this change is not observed in PE [16, 20]. So, the levels of inflammatory cytokines in maternal serum increase and the initial conditions would be prepared for the activation of immunosuppressive mechanisms in MSCs. Understanding the mechanisms through which these cells regulate immune system is essential for their use in immunotherapy and clinical applications for different clinical conditions, including preeclampsia.

Transforming Growth Factor-Beta (TGF-β) and NO are key regulatory factors secreted by MSCs and altered levels of these factors have been reported in patients with preeclampsia. In the present study, to better identify the potential role of MSCs in the alterations of TGF-β and NO levels in preeclampsia, TGF-β and NO secretion by adipose-derived MSCs were compared between pregnant preeclamptic and normal pregnant women.

## Materials and Methods

### Adipose tissue collection

The adipose tissues were collected from 10 preeclamptic patients and 10 age-matched normotensive controls at the time of cesarean section delivery. Prior to tissue collection, informed consent was obtained from the participants. Demographic and clinical data of the participants are given in Table 1. The study protocol for tissue collection was approved by the Ethics Committee of Shahed University (Shahed.REC.1392.7). The diagnosis of PE was made based on the definitions of the American College of Obstetricians and Gynecologists [21]. It was defined as the presence of hypertension and proteinuria after the 20th week of pregnancy. Blood pressure elevation with systolic blood pressure ≥140 mmHg or

**Table 1.** Comparison of demographic and obstetric parameters in women with preeclampsia and normal pregnancies

| Variable | (n=10) | | P |
|---|---|---|---|
| | Normal Pregnancy | Preeclampsia | |
| Maternal age (years) | 28.6±3.6 | 28.1±5.2 | 0.8 |
| Gestational age at delivery (weeks) | 39.1±1.3 | 37.4±0.96 | 0.004* |
| Birth weight (g) | 3374±422.27 | 2745±628.26 | 0.002* |
| Systolic blood pressure(mmHg) | 114.5±11.65 | 155±12.7 | <0.001* |
| Diastolic blood pressure(mmHg) | 77±8.23 | 99.5±8.95 | <0.001* |

P-value refer to comparison of the 2 groups by the Mann-withney U-test. IMMUNOREGULATION

diastolic pressure ≥90 mmHg was considered hypertensive. Also, proteinuria of >300 mg per 24 h collection or a minimum of 1+ on a dipstick was considered to be significant. A minimum of two consecutive measurements were carried out for the diagnosis. Exclusion criteria were fetal structural or genetic anomalies, maternal chronic hypertension, complicated forms of PE, such as HELLP syndrome (hemolysis, elevated liver enzymes, and low platelets), cardiovascular disease, renal disease, hepatic disease, diabetes, or any infectious disease.

## Isolation of MSCs

Isolation and culture of adipose derived MSCs were performed following the method described by Bunnell et al. with some modifications [22]. Phosphate-Buffered Saline (PBS) was used to wash the adipose tissue several times so as to remove excess blood. After cutting the tissues into small pieces (1cm3) using scissors, they were incubated with 0.075% type I collagenase (Gibco®) for 30-45 min and then mononuclear cells in the collagenase were collected using centrifugation. The cells pellets were washed with PBS and resuspended in Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL, Life Technologies, Merelbeke, Belgium) supplemented with 10% fetal bovine serum (Sigma) and seeded into a T-75 flask. Next, the cultures were maintained at 37ºC in a humidified atmosphere with 5% $CO_2$. Three to five days after the incubation, the small digested residues were removed and the culture was continued for two month. Meanwhile, the medium was replaced once or twice per week, every three to four days.

## Flowcytometric analysis

From the second passage, the specific surface antigens of MSCs in the cultures were characterized using flow cytometric analysis. The cells were harvested making use of trypsin digestion and stained with Fluorescein Iso ThioCyanate (FITC)- or PhycoErythrin (PE)-conjugated antibodies against CD14, CD34, CD44, CD45, HLA-DR, CD73, CD90, and CD105. Finally, the immonophenotyping analysis was evaluated using a FACSCalibur instrument (Becton Dickinson). Data was analyzed using Cyflogic software (CyFlo Ltd.).

## Evaluation of differentiation

### Adipogenic induction

The medium was replaced with adipocyte induction medium or control medium when cells reached 70% confluence, according to the method described by Bunnell et al. [22]. The induction medium contained a low glucose Dulbecco's Modified Eagle's Medium (LG-DMEM) with 10% FBS, 100 µ Mindomethacin, 5 µM insulin, 0.1 µM dexamethasone, 0.5 mM IsoButyl Methyl Xanthine (IBMX), 100 U/ml penicillin, and 100 mg/ml streptomycin. After 72 h, the medium was changed to adipocyte maintenance medium, that was identical to the induction media, but without IBMX. The cells were maintained in the culture for 14 days, with 90% of the maintenance media replaced every three days.

## Osteogenic induction

The medium was replaced with osteoblast induction medium or control medium, as described by Bunnell et al. [22]. Osteoblast induction medium contained DMEM (low glucose) with 10% FBS, 10 mM b-glycerophosphate, 50 µg/ml ascorbate-2-phosphate, 10 nM dexamethasone, 100 U/ml penicillin, and 100 mg/ml streptomycin. The cells were maintained in culture for 21–28 days, with 90% of the media replaced every 3 days.

## TGF-β and NO assay

After the second passage and suspension, MSCs were counted and 10,000 cells were cast in 24-well plates. Af-

ter a few days, when the cells reached 80-90% confluence, cell supernatants were collected and centrifuged (480 g for 5 min). The cell supernatant was stored at -70°C. TGF-β concentration was measured using a commercial ELISA kit (R&D System, USA) and NO level was measured using Griess method. All the tests were performed according to the manufacturer's guidelines.

### Statistical analysis

The data was analyzed running the student t-test and Mann-Whitney test in SPSS V. 19. A Probability (P) value of less than 0.05 was considered to be statistically significant.

## Results

Demographic and clinical data of the participants are summarized in Table 1. In the preeclamptic group, the gestational age at delivery and birth weight were significantly lower than those of the control group. Systolic and diastolic blood pressures were significantly higher in women with PE than in the normal controls.

### Isolation and characterization of adipose derived mesenchymal stem cell

The adipose tissue was enzymatically digested and cultured. This separation was performed based on the adhesion properties of MSCs to culture dishes. In subsequent passages, MSCs were morphologically purer and more

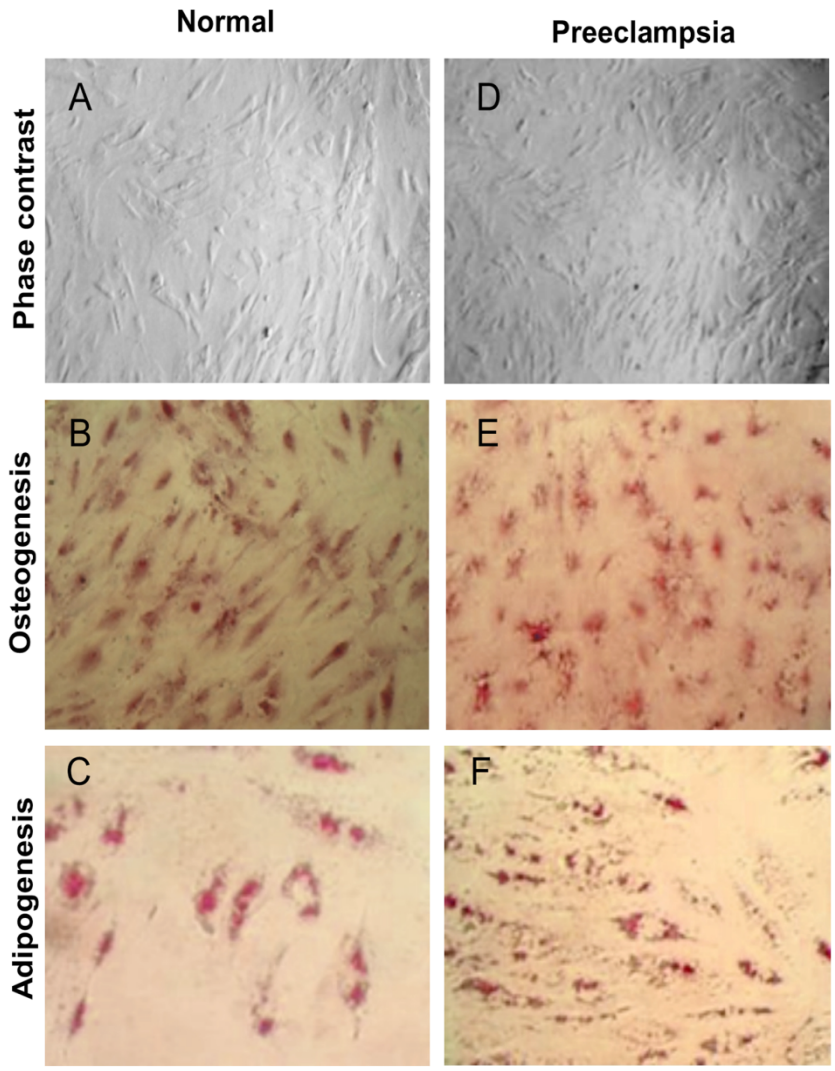


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**Figure 1.** Morphology and differentiation of isolated adipose derived mesenchymal stem cells at passage 2( x20) in women with preeclampsia and normal pregnancies showed fibroblastoid cells (A, D).Calcium deposition as Osteogenic differentiation were verified by Alizarin Red staining (B, E) and Adipogenic differentiation were verified by Oil Red O staining (C, F).

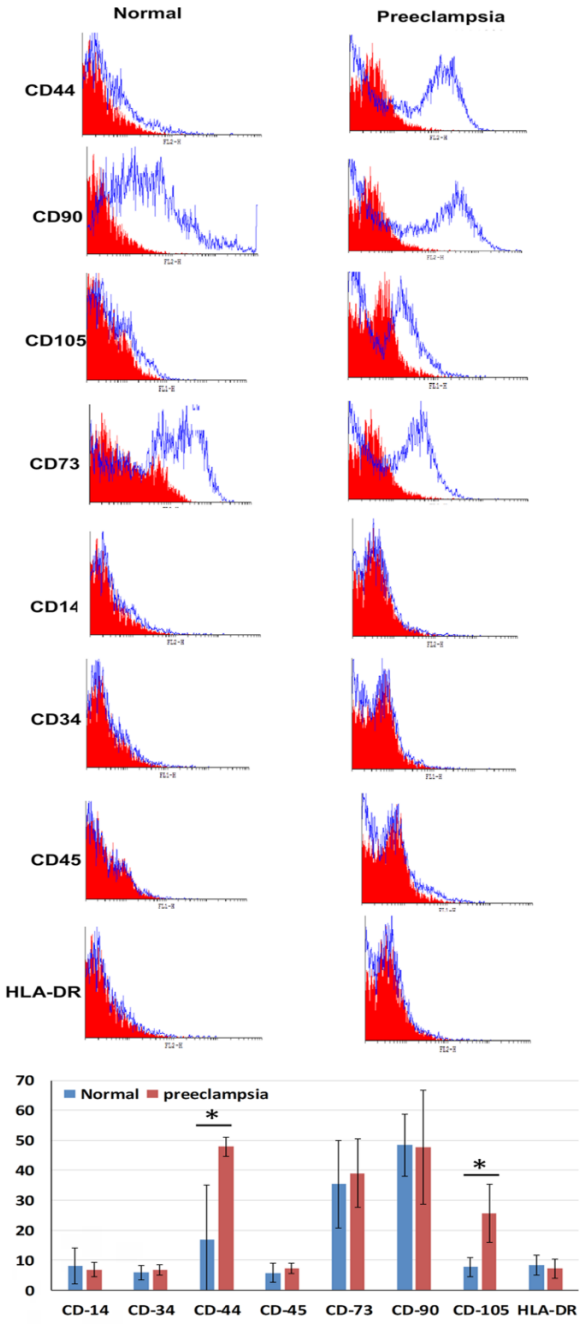


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**Figure 2.** Flow cytometric analysis of isolated adipose derived mesenchymal stem cells at passage 2 in women with preeclampsia (Right panel) and normal pregnancies (Left panel) Immunophenotypic markers. Red and blue histogram, displayed isotype control anti-bodies labeling and surface proteins, respectively. Data are shown as the Mean±SD.

similar to fibroblasts (spindle-shaped). The morphological characteristics of the cells under inverted microscope are illustrated in Figure 1 (A, D).

## Differentiation of adipose-derived MSCs

To evaluate the ability of differentiation into adipogenic and osteogenic lineages, the MSCs were cultured in a special differentiation medium. At the end of this period, in osteogenic differentiation wells, some compact and well-established areas were observed, such as nodules and in fat differentiation wells, lipid vacuoles were noticed. At the end of cell differentiation period, Alizarin Red (Figure1 B, E) and Oil red O (Figure 1 C, F) staining were performed.

## Immunophenotyping of adipose derived MSCs

Another way to prove the nature of the MSCs is evaluating the expression of surface markers using

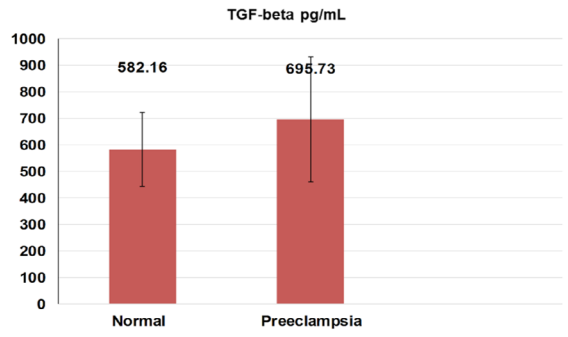


**Figure 3.** TGF-β1 production by Adipose derived mesenchymal stem cells at passage 2. Data are shown as the Mean±SEM.

flowcytometry. Adipose derived MSCs in preeclamptic and normal pregnant women expressed CD105, (SH2/Endoglin), CD73 (SH3), CD44, and CD90 (Thy-1). In contrast, CD45 (leukocytes marker), CD34 (hematopoietic stem cell marker), CD14 (macrophage marker), and HLA-DR markers had little expression on the surface of the cells (Figure 2).

## Measurement of TGF-β release in adipose-derived MSCs

After the second passage, 10000 MSCs were cultured. After 3-5 days of cell proliferation, cell supernatants were collected and TGF-β1 assay was performed using ELISA technique. The mean level of TGF-β1 secreted by adipose derived MSCs was 582.16±2.35 pg/mL in healthy pregnant women and 695.73±3.92 pg/mL in women with PE (Figure 3), revealing a significantly higher level in the former group (P=0.03).

## Measurement of NO release in adipose-derived MSCs

After the second passage, 10000 MSCs were cultured. After 3-5 days of cell proliferation, cell supernatants were collected and NO level was measured following Griess method using ELISA technique. The mean level of NO secreted by adipose derived MSCs was 0.5±0.03 pg/mL in healthy pregnant women and 0.57±0.04 nmol/mL in women with PE (Table 2), revealing no significant change between the two groups (P=0.81).

## Discussion

In the current study, subcutaneous adipose tissue derived MSCs were isolated from a number of preeclamptic and healthy pregnant women during cesarean section, and then after amplification and purification, their ability to differentiate into bone and fat was examined using specific differentiation media. In both groups, the ability of differentiation into adipocyte and osteocyte showed no differences in terms of the characteristics. Flow cytometric analysis showed the expression of CD90, CD73, CD44, and CD105 markers and no expression for CD-14, CD34, CD45, and HLA-DR markers.

Rolfo et al. investigated immunophenotype of placenta-derived MSCs of both normal pregnant and preeclamptic women and showed that these cells were positive for CD105, CD166, CD90, and CD73 while they were negative for HLAII, CD34, CD133, CD20, CD326, CD31, CD45, and CD14 [8]. Also, Wagner et al. studied immunophenotyping of MSCs derived from bone marrow, adipose tissue, and umbilical cord blood and showed that MSCs of all these three sources were positive for the expression of markers such as CD10, CD14, CD24, CD31, CD34, CD36, CD38, CD45, CD49d, CD117, and CD133 and negative for the expression of markers such as CD13, CD29, CD44, CD73, CD90, CD105, CD166, and HLA-ABC [23]. The results of immunophenotyping in the present study are consistent with the results reported in these studies.

Our results showed that TGF-β secretion by MSCs in preeclamptic women was significantly higher than that in control group. Despite the exhaustive number of studies on the secretion of TGF-β by human MSCs [24], no study was found examining the secretion of TGF-β by these cells in normal and preeclamptic pregnancies.

Lim et al. measured plasma levels of soluble Endoglin-1 (Eng-1) and TGF-β in the second trimester of pregnancy and showed that in PE, TGF-β decreased and

**Table 2.** NO production by Adipose derived mesenchymal stem cells at passage

| Groups | N | Mean±SEM | P |
|---|---|---|---|
| Normal | 10 | 0.5±0.03 | 0.81 |
| Preeclampsia | 10 | 0.57±0.04 | |

P-value refer to comparison of the 2 groups by t-test.

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Eng-1 increased, and concluded that this change could be associated with the severity of disease [25]. On the other hand, by measuring the plasma level of TGF-β in the third trimester of pregnancy, Paracoli et al. showed that the level of this factor increased significantly in PE as compared with that in normal pregnancy demonstrating that changes in this factor shows its involvement in the pathophysiology of the disease [26]. Also, Wang et al. measured TGF-β in the chorionic villi and reported that the secretion of this factor increased more significantly in PE than in normal pregnancy. Changes in this factor could suggest its role in the pathophysiology of the disease and vascular endothelial injury [27]. The difference between the studies cited may be due to the differences in sampling time, type and number of samples, and techniques used.

The role of TGF-β family members has been confirmed in the biological processes, embryonic development, adult tissue homeostasis, as well as pathogenesis of some diseases, such as cancer, autoimmune diseases, cardiovascular diseases, and fibrosis [26]. Some of the effects of TGF-β secreted by MSCs include suppressing the functions of natural killer cells and cytotoxic T cells, inducing mononuclear cells proliferation in peripheral blood, and increasing the number of regulatory T cells [24, 28]. TGF-β has effects on a variety of cells, but its main role is regulating immune responses through stimulating tolerance and inhibiting inflammation. It is assumed that cytokine balance changes in the cell environment can alter the effects of this factor and may play a role in immunopathology [29].

In PE, increased expression of Th1 cytokines and decreased expression of Th2 cytokines indicates the shift of Th1/Th2 balance toward Th1-responses [30]. Given that MSCs often play a mediating role in down-regulation of Th1 proinflammatory Cytokine, increased secretion of TGF-β by these cells in preeclamptic group can be an attempt to suppress inflammatory responses. Therefore, MSCs-based therapy may regulate Th1/Th2 balance in this condition. Also, according to the results of the present study, NO secretion by MSCs was not significantly different in the two groups. Although an exhaustive number of studies are reported on the secretion of NO by human MSCs [31], no study was conducted on the secretion of NO by these cells in normal and preeclamptic pregnancies.

Kukor et al. showed that reduction of serum levels of NO in preeclamptic women may be due to reduced affinity of Endothelial NO Synthase (eNOS) to its cofactor, increased production of superoxide anion, and increased oxidative stress [32]. Reduction of NO causes endothelial dysfunction, vascular constriction, and less invasive trophoblas [33]. Moreover, Shaamash et al. reported that increased serum levels of NO in preeclamptic women could indicate compensatory and conservatory role of this mediator in maintaining feto-maternal circulation and decreasing platelet adhesion [34]. The difference between these studies may be due to differences in diet, drug interactions, and urinary excretion [35].

The results of the previous studies suggest that TGF-β directly dilates vessels by eNOS. In patients with PE, it increases Soluble Endoglin in peripheral blood circulation, inhibits binding of TGF-β to its receptor, and consequently, suppresses the activity of eNOS and vascular dilation and thereby increases blood pressure [36, 37]. In addition to its hypertensive effect, TGF-β plays a role in proteinuria of PE,too. Findings of the studies on the etiology of kidney damage in PE shows that Ang-II, together with TGF-β and endothelin, activate collagen type I gene and therefore increase the formation of extracellular matrix in renal cortex. In fact, Ang-II stimulates the expression of TGF-β in kidney and endothelium and increases the chorionic villi of preeclamptic placentas [38, 39].

We have shown that the secretion of TGF-β by adipose derived MSCs is different in preeclamptic and healthy pregnant women. Further studies need to understand the mechanism for the different secretion of this factor in both groups. Data obtained in the present study, along with those of the previous studies, suggested that MSCs may play an important role in immunoregulation. Therefore, MSCs-based therapy may regulate Th1/Th2 balance in PE. Inversely, we have shown that the secretion of NO by adipose derived MSCs of preeclamptic and healthy pregnancies were not significantly different. Therefore, it can be concluded that NO secreted by MSCs may have no significant role in the pathology of PE. Further investigations are recommended to examin the amount of NO secreted by MSCs derived from other tissues, such as chorionic villi of the placenta, in the two groups

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