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Original Article

LMWH inhibits TNF-α and IL-6 in placental villous explants and its effects are attenuated by TLR-4/NF-κB p65 blocking in JEG-3 cells

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Abstract: Preeclampsia (PE) leads to high perinatal mortality and morbidity due to hypertensive disorder and maternal inflammatory response of pregnancy. Studies demonstrated higher levels of TNF-α and IL-6 were observed in cases of PE. This research aims to explore the effects of low molecular weight heparin (LMWH) on TNF-α and IL-6 and its preliminary potential mechanism through TLR-4/NF-κB p65 pathway in placental villous explants and JEG-3 cells. Placental villous explants derived from patients with PE, spontaneous preterm labor and first trimester termination of pregnancy were cultured under normoxia and hypoxia conditions respectively. Then different concentrations of LMWH were added to explants in hypoxia group. In terms of gene target study, JEG-3 cells were transfected by SiRNA against TLR-4 and NF-κB p65 individually. Protein and mRNA levels of TNF-α and IL-6 were measured by western-blot and PCR. Immunohistochemistry staining was used to locate relative proteins in placental villous explants. Placental villous explants under hypoxia condition had similar upregulated levels of TNF-α and IL-6 with that in PE group, and this upregulation could be inhibited by LMWH with 2.5 μg/ml as the most effective concentration. TLR-4 and NF-κB p65 were both higher expressed in PE group than those in control group. Blocking of TLR-4 or NF-κB p65 in JEG-3 cells could both lead to attenuation of anti-inflammatory properties of LMWH. The results suggested LMWH could inhibit inflammatory response in placenta villous explants by inhibiting TNF-α and IL-6 and the effects were possibly via TLR-4/NF-κB p65 pathway.

Keywords: Inflammatory factor, low molecular weight heparin, placental villous explants, preeclampsia, TLR-4/NF-κB p65

Introduction

Preeclampsia (PE) is a potentially life-threatening disorder of pregnancy characterized by hypertension and proteinuria. It results in consequence of substantially perinatal mortality and morbidity of mothers and infants with an occurrence of approximately 2% to 10% of pregnancies [1]. Genetic factors, placental oxidative stress, endothelial dysfunction, as well as immune tolerance imbalance, are believed to contribute to etiology of PE. Nevertheless, specific pathological mechanism from these potential factors to PE has not been fully understood yet which needs to be studied further.

Recently an increasing amount of data suggested enhanced systemic and local maternal inflammatory response played a crucial role in etiology of PE. A markedly elevating quantity of inflammatory factors were detected in patients with PE compared with normal pregnancy [2-4]. Higher levels of TNF-α and IL-6 derived from maternal serum and placenta were observed in patients with PE [5, 6]. Also hypoxic placenta was thought to be a promotion on synthesis of TNF-α and IL-6 [7] and could induce a PE-like inflammatory response [8].

Low molecular weight heparin (LMWH) has been found to be effective and safe for prevention and therapy of PE [9, 10]. Anticoagulant mechanism of LMWH seems to be responsible for the curative effectiveness. Nevertheless, recent studies suggested LMWH may exert anti-inflammatory properties, which was independent of its anticoagulant activity [11-13]. The mechanism of this anti-inflammatory effect

of LMWH applied to treatment of PE has not been investigated yet.

Except major events leading to PE, which are failure of implantation and transformation of spiral artery during the first half of pregnancy, hypoxia of placenta is also an important reason for causing PE. In normal physiological conditions, early placental and embryo development occur in an environment of physiologically low oxygen, but there is a rise of oxygen tension within the placenta towards the end of pregnancy. Normal concentration of intervillous oxygen at first trimester stage is approximately 8% and the concentration in hypoxia condition is 2% or even lower [14, 15].

It has been shown Toll-like receptor-4 mediated nuclear factor κB (TLR-4/NF-κB) signaling plays a critical role in secretion of inflammatory cytokines [16]. Hasan Babazada et al. found that self-assembling lipid modified glycol-split heparin nanoparticles suppressed lipopolysaccharide-induced inflammation through TLR-4-NF-κB signaling [17]. Therefore, it gives the idea that LMWH perhaps carries out its anti-inflammatory function via TLR-4/NF-κB pathway, which is preliminarily investigated in this paper.

Therefore, this study aims to firstly verify hypoxia of placenta can produce the similar pathological inflammatory condition compared with PE patients by measuring TNF-α and IL-6 expression in placental explants. Then the regulation function of LMWH on TNF-α and IL-6 and its preliminary mechanism via the TLR-4/NF-κB p65 pathway were respectively studied in placental explants and JEG-3 cells.

Materials and methods

Firstly, placental explants were collected to validate the higher levels of TNF-α and IL-6 in PE. Hypoxia-induced consequence was then studied to illustrate hypoxia could produce the similar pathological inflammatory condition compared with PE patients. And we utilized JEG-3 cells to probe into the target of LMWH by small interfering RNA (SiRNA) against TLR-4 and NF-κB p65 in vitro.

Samples collection

Placental tissues were obtained from the obstetrics ward of the affiliated hospital of Qingdao University medical college with approval of the research ethics board. Informed consent was obtained from all individual participants included in the study. The recruited patients were classified into the following three groups and each group enrolled 20 patients:

(1) PE group: severe PE, blood pressure >160/110 mmHg coupled with proteinuria in urine collection (>2 g per 24 h).

(2) Control group: spontaneous preterm labor who had the similar gestational ages with PE group. This group was used for comparing the increasing expression of inflammatory factors with PE group without any hypoxic stimulus.

(3) Hypoxia group: first trimester termination of pregnancy (11-13 weeks) due to the personal willingness of patients. This group giving a hypoxia condition were chosen to mimic the similar state of PE model.

Patients in PE group and 8 patients in Control group were all underwent cesarean section without labor pains. The other 12 patients in Control group were natural births with labor pains. These enrolled subjects needed to get through the temperature monitoring and blood routine examination to exclude the general infection factors. All samples were obtained by C-section or normal delivery or artificial abortion under sterile conditions (see **Table 1**).

Placental explants culture

All placentas explants were derived from each patient's placenta and each placenta was divided into several placentas explants. Placentas were dissected in sterile cold PBS and transferred to lab within 1 h, then cultured in warm Dulbecco's Modified Eagle's Medium/Ham's F-12 Medium (GIBCO, USA), and concrete method was same as previously described [18]. Secondly, basal plate, chorionic plate and infarcted regions were removed from the placentas after washing by PBS, then the remaining parts were dissected into explants (weight range: 10-30 mg). Around total wet weight of 300 mg were cultured in 24-well culture plates and each well was filled with 1 mL serum-free culture medium. Placenta villous explants from hypoxia group were cultured under normoxia and hypoxia conditions both at 37°C, which were 5% CO_2 and 8% O_2, 5% CO_2 and 2% O_2 respectively. Oxygen was controlled and regu-

Table 1. Clinical characteristics of patients in three groups

	PE group (n=20)	Control group (n=20)	Hypoxia group (n=20)
Maternal age (yrs)	30.43±3.51	32.00±2.58	25.00±3.16[A,B]
Gestational age (yrs)	35.23±0.46[A]	33.04±0.87	12.50±0.52[A]
Systolic blood pressure (mmHg)	122.29±7.45[A]	170.14±13.19	117.20±8.32[A,B]
Diastolic blood pressure (mmHg)	77.43±3.99[A]	119.86±8.41	76.00±4.95[A]

[A]p<0.05, vs control group; [B]p<0.05, vs control group.

Table 2. SiRNA sequences

	Forward	Backward
TLR-4	5'-GGAAUGAGCUAGUAAAGAATT-3'	5'-UUCUUUACUAGCUCAUUCCTT-3'
NF-κB p65	5'-GGAGUACCCUGAGGCUAUATT-3'	5'-UAUAGCCUCAGGGUACUCCTT-3'
Scramble	5'-UUCUCCGAACGYGUCACGUTT-3'	5'-ACGUGACACGUUCGGAGAATT-3'

lated by input of air and CO_2 in incubator. In PE group and control group, placenta villous explants only needed to be cultured under normoxia condition.

Placentas explants from hypoxia group were cultured without or with different concentrations of LMWH under self-oxygen tension for a maximum period of 24 h, at which point explants were collected for analysis at a later stage. LMWH sodium from the same batch in concentrations of 0.25, 2.5 and 25 μg/ml in culture medium were respectively added to the placentas explants in this experiment.

JEG-3 cell cultures

JEG-3 cells were purchased from Shanghai cell bank and cultured in MEM supplemented with 50 mg/ml gentamicin, penicillin/streptomycin and 10% fetal calf serum at 37°C in normoxia and hypoxia conditions, which were 5% CO_2 and 21% O_2, 5% CO_2 and 2% O_2 respectively. It is worth noting that 21% O_2 and 8% O_2 conditions are normal oxygen concentrations for the JEG-3 cells and villous explants respectively.

SiRNAs against TLR-4 and NF-κB p65

SiRNAs against TLR-4 or NF-κB p65 were designed and synthesized by Ambion Corporation (as shown in **Table 2**). JEG-3 cells cultured in a 6-well plate were transfected with 200 nM siRNA or scramble using Lipofectamine 2000 (Invitrogen) according to the instructions. Western-blot was performed to evaluate the efficiency at 48 h after transfection. Cells were plated in 6-well dishes (1×10^6 cells/well) and incubated overnight. The medium was replaced by fresh medium in the absence or presence of 2.5 μg/ml of LMWH under normoxic and hypoxic conditions in the following day. After 24 h, cells were collected for further use.

Immunohistochemistry staining

Immunohistochemistry was performed to locate antibodies of TNF-α, IL-6, TLR-4 and NF-κB p65 (from Santa Cruz) in placentas tissues. Secondary antibodies and DAB reagents from the Power Vision™ Two-Step Histostaining Reagent kit (Beijing Zhongshan Biotechnology Co., Ltd, China) were used according to manufacture instruction. Immuohistochemical results were pictured by light microscopy (CX31, Olmpus, Japan) equipped with a camera.

Real-time reverse transcriptase-PCR

Total RNA was extracted from placenta explants or JEG-3 cells using Trizol and reverse-transcribed using High Capacity cDNA Archive Kit (Takara). Semiquantitative real-time polymerase chain reaction was performed to quantify mRNA levels of TNF-α and IL-6 with β-actin as housekeeping gene. All the primers were designed using Primer 5.0 by Sangon Biotech (Shanghai) Co., Ltd (as shown in **Table 3**). Data was expressed as the percentage change in gene expression compared to control samples using the $2^{-\Delta\Delta Ct}$ method.

Western-blot

Total proteins were extracted from 6 placenta explants in each group respectively and JEG-3 cells by adding an appropriate volume of RIPA buffer. About 20 g protein was separated under reducing conditions by SDS-PAGE, transferred onto PVDF paper and incubated with primary antibodies against human TNF-α and IL-6 at 4°C overnight. Then the membranes were incubated for 2 h with second antibody after washing with TBST. Immunoreactivity was detected by chemiluminescence immunoassay. Relative intensities of bands were determined by densi-

Table 3. Real-time PCR Primers

	Forward	Backward
TNF-α	5'-CACCATGAGCACTGAAAGCA-3'	5'-GGTTCGAGAAGATGATCTGACTG-3' 252 bp
IL-6	5'-GAGTAGTGAGGAACAAGCCAGAG-3'	5'-TTGGGTCAGGGGTGGTTAT-3' 106 bp
β-actin	5'-TAGTTGCGTTACACCCTTTCTTG-3'	5'-TCACCTTCACCGTTCCAGTTT-3' 151 bp

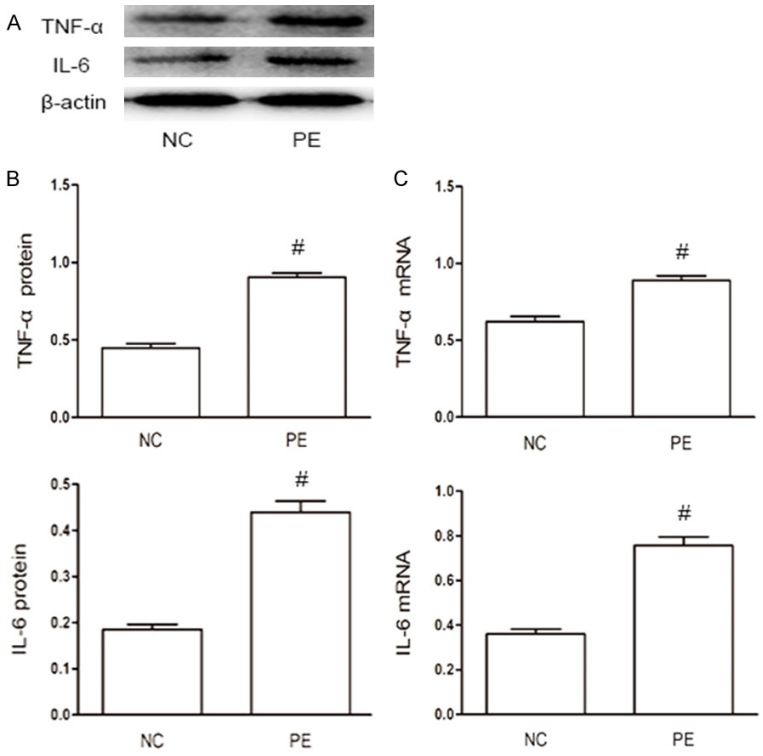

Figure 1. Protein and mRNA expressions of TNF-α and IL-6 in placenta from normal control (NC) and PE groups (PE). A. Protein expressions of TNF-α and IL-6 were measured by western-blot, with β-actin as housekeeping gene (n=5); B. Relative grayscale ratios of **Figure 1A**; C. Relative levels of mRNA of TNF-α and IL-6 were measured by RT-PCR (n=5). #p<0.05, vs NC group.

tometry using Fusion-capt 16.08 a software. β-actin was used as an internal control for TNF-α and IL-6.

Statistic analysis

Data were presented as mean ± standard deviation and analyzed using SPSS17.0 software. Mann-Whitney test was used to compare the difference between two groups. P<0.05 was considered to be significant.

Results

Elevated mRNA and protein levels of TNF-α and IL-6 in PE and hypoxia group

Both protein and mRNA levels of TNF-α and IL-6 in placenta explants were significantly increased in PE group compared with those in control group (**Figure 1**). Placenta explants in hypoxia group under normoxia condition (8% O_2) had almost the same protein and mRNA levels of TNF-α and IL-6 with control group (**Figures 1** and **2**). However, under hypoxia case (2% O_2), protein and mRNA levels of TNF-α and IL-6 elevated remarkably (36.3%-82.2%), which was close to that in PE group (**Figure 2**), indicating the successful mimic to the placenta physical environment in patients with PE.

LMWH inhibited the upregulation of TNF-α and IL-6 induced by hypoxia

Hypoxia-mediated up-regulation of mRNA and protein of TNF-α and IL-6 were significantly inhibited by LMWH, with 2.5 μg/ml as the most effective concentration (P<0.05). However, higher concentrations had no additional inhibitory effect as showed in **Figure 2**. Based on this result, all following gene target experiment in JEG-3 cells only employed concertation of 2.5 μg/ml.

Increased expressions of TNF-α, IL-6, TLR-4 and NF-κB p65 in placetas of PE

As shown in **Figure 3**, TNF-α and IL-6 mainly expressed in cell membrane and cytoplasm of syncytiotrophoblast in villous structures from PE group compared with control group (**Figure 3A-D**). Also, we found a significant increase of expressions of TLR-4 and NF-κB p65 in PE group (**Figure 3E-H**). TLR-4 expressed mainly in cytoplasm of syncytiotrophoblast and NF-κB p65 expressed in cytoplasm and nucleus of syncytiotrophoblast, which were both heavi-

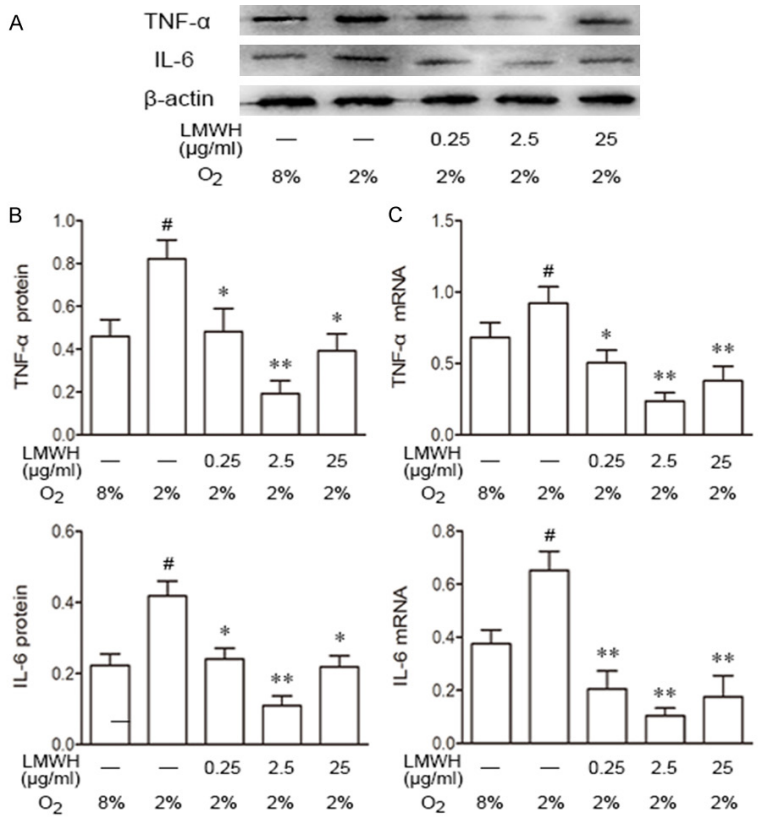

Figure 2. Protein and mRNA expressions of TNF-α and IL-6 in placenta explants villous from first trimester stage incubated with different concentrations of LMWH under 8% O_2 and 2% O_2. A. Protein expressions of TNF-α and IL-6 were measured by western-blot, with β-actin as housekeeping gene (n=6); B. Presents the relative grayscale ratios of **Figure 2A**; C. Relative levels of mRNA of TNF-α and IL-6 were measured by RT-PCR (n=6). #p<0.05 *vs* 8% O_2, *p<0.05, **p<0.01 *vs* 2% O_2 and none LMWH.

er stained in PE group than those in control group.

LMWH inhibited hypoxia-induced upregulation of TNF-α and IL-6 in JEG-3 cells, and the effects were attenuated after blocking TLR-4 or NF-κB p65

Firstly, SiRNA transfected effectiveness was verified by measurement of TLR-4 and NF-κB p65 expressions, compared with before and scramble transfection, as showed in **Figure 4A**. Both of TLR-4 and NF-κB p65 expressions significantly decreased (P<0.05). We found that hypoxia-mediated upregulation of TNF-α and IL-6 were significantly inhibited by LMWH in JEG-3 cells (P<0.05, **Figure 4B**), just as in placenta explants. However, silencing of TLR-4 or NF-κB p65 gene led to attenuation of the effect of LMWH on hypoxia-induced increase of TNF-α and IL-6 protein expressions as showed in **Figure 4C**, **4D**. These results indicated that LMWH exhibited an anti-inflammatory effect in human trophoblast cells possibly via TLR-4/NF-κB p65 pathway.

Discussion

It is well-established that LMWH is eutherapeutic in patients with PE though few researches demonstrated LMWH has little impact [19-21]. Studies indicated LMWH had ability to prolong gestational weeks, improve neonatal prognosis, reduce maternal morbidity and mortality and its safety and efficacy had been confirmed in clinical practice [22, 23]. Originally, LMWH is known to have a wide variety of anticoagulant effects, as thromboembolism presenting in PE. Futhermore, it is proved that LMWH is also effective for patients without prothrombosis [24].

LMWH also plays important roles in protecting vascular endothelial cells, anti-inflammation, anti-apoptosis, immunoregulation and promoting cytotrophoblast invasiveness and so forth [25]. Although the etiology of PE is still unclear, inflammation has been confirmed contributing to pathogenesis of PE, which were proved by the fact that elevated levels of pro-inflammatory cytokines could drive the cytokine network in preeclamptic women towards an excessive systemic inflammatory state [26]. LMWH was reported to attenuate inflammation, as some in vitro and in vivo studies in rats shown that it could decrease plasma levels of pro-inflammatory cytokines [27]. Here, this paper aims to explore the preliminary anti-inflammatory effects of LMWH in PE.

Pinheiro MB found IL-6 and IFN-γ levels in maternal circulation were higher in women with PE compared to normal pregnancies. This study also found TNF-α and IL-6 levels were higher in placenta villous explants from PE women than normal pregnant controls. Lee SM reported

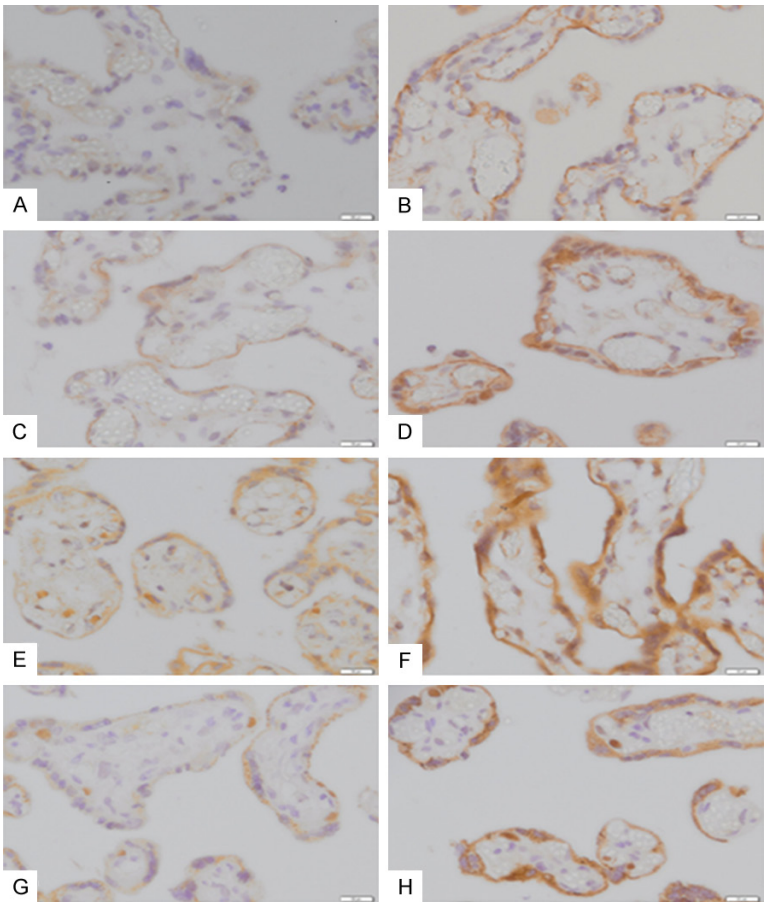

Figure 3. Location and expressions of TNF-α, IL-6, TLR-4 and NF-κB p65 in placenta explants. Localization of TNF-α, IL-6, TLR-4 and NF-κB p65 in placentas of each group were examined by immunohistochemistry (×400, n=4). Brown deposits indicated positive staining. A. TNF-α in control group; B. TNF-α in PE group; C. IL-6 in control group; D. IL-6 in PE group; E. TLR-4 in control group; F. TLR-4 in PE group; G. NF-κB p65 in control group; H. NF-κB p65 in PE group.

that a more intense and rapid inflammatory response of peripheral blood mononuclear cells was observed with microparticles from hypoxic trophoblasts compared to microparticles from normal trophoblasts [28]. This research employed this hypoxia idea and results showed that TNF-α and IL-6 levels increased under hypoxia conditions both in placenta explants and JEG-3 cells.

After confirming the hypoxia model, next step was to investigate the beneficial effect of LMWH to PE. Hochart H et al. found treatment with pharmacological doses of LMWH and unfractioned heparin significantly attenuated LPS-induced production of TNF-α, IL-8, IL-6 and IL-1β as well as NF-κB translocation in human monocytes [29]. Mulla M et al. reported heparin can attenuate the anti-β2GPI antibody-mediated pro-inflammatory response in first trimester trophoblasts, but only at high concentrations [30]. Here, in our *in vitro* experiments, incubation with LMWH, either in placenta explants from preeclamptic patients or from normal pregnant controls, as well as in JEG-3 cells, led to a significant attenuation in hypoxia-induced increase of protein expressions of TNF-α and IL-6, compared to no LMWH incubation. The most effective concentration, which induced the strongest decrease in inflammation was 2.5 μg/ml. As data showed in **Figure 2**, there was a reverse in terms of both mRNA and protein expressions of TNF-α and IL-6 under 25 μg/ml LMWH condition. In the rage of LMWH concentration during the experiment in this research, preliminary experiment showed no obvious cytotoxicity. Then speculation refers to this reverse was LMWH has different action targets perhaps. One action target generated effect in the scope of relative low LMWH concentration and its impact improved with the increase of LMWH concentration within the low threshold. Up to the optimal inhibition effect, the adding concentration would activate another target, meanwhile, disturb or even inhibit the first target. However, this is just our own hypothesis and specific mechanism needs to be investigated by subsequent design tests. The modality of the anti-inflammatory effects of LMWH has not yet been fully clarified, but could be mediated through ability of heparin to bind and inactivate proteins such as growth factors, and adhesion molecules, such as L- and P-selectins involved in recruitment of inflammatory cells [31]. Furthermore, heparin maybe inhibit adhesion and migration of leukocytes in endothelium by binding to cell surface proteins, such as β2-integrin adhesion molecule CD11b/CD18 and platelet/endothelial cell-adhesion molecule 1 [32].

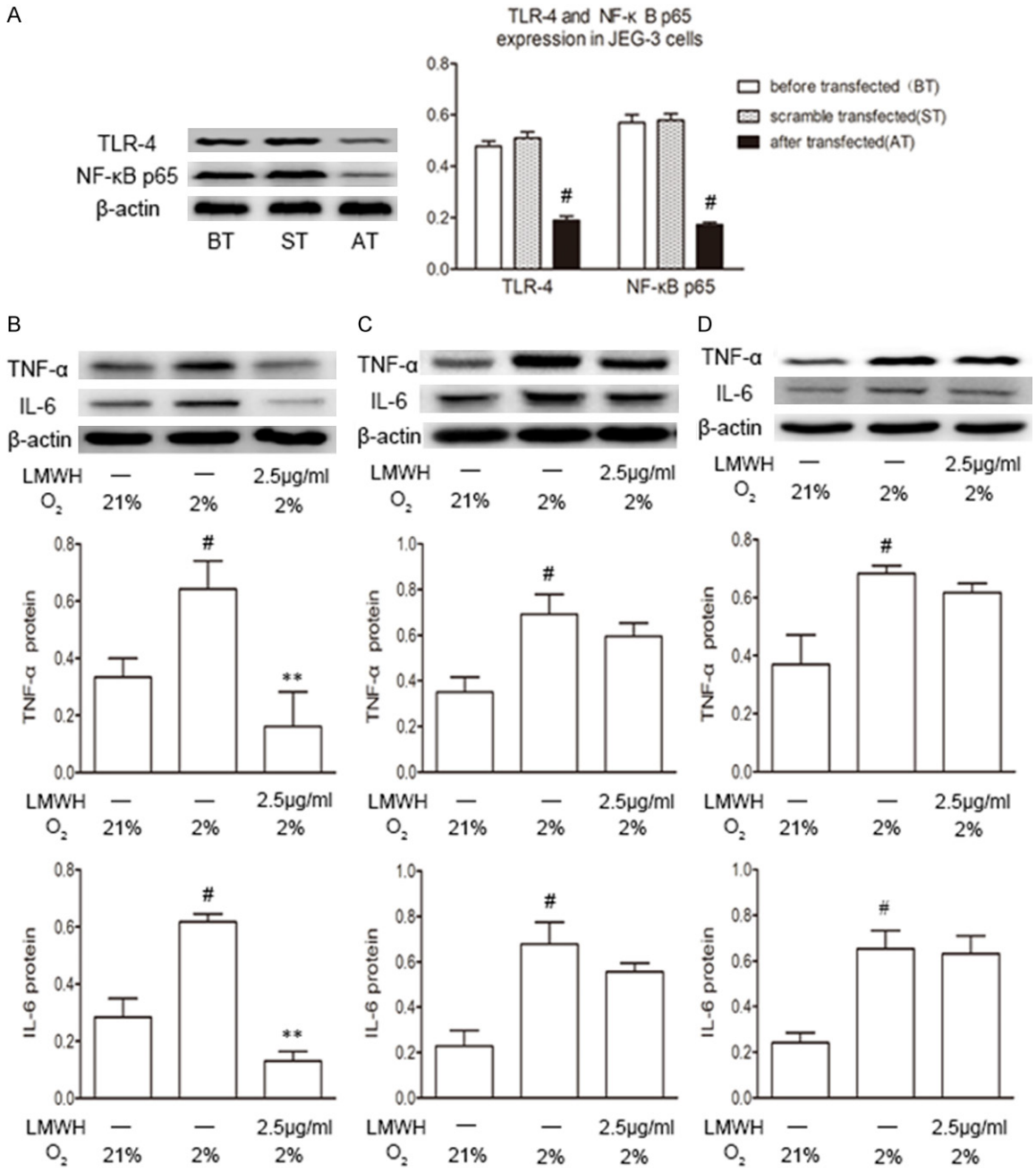

Figure 4. Protein expressions of TNF-α and IL-6 under 21% O_2 and 2% O_2 with or without 2.5 μg/ml LMWH in JEG-3 cells, cells were silenced TLR-4 or NF-κB p65 gene respectively. A. JEG-3 cells were silenced TLR-4 or NF-κB p65 gene respectively, expressions and relative grayscale ratios of TLR-4 and NF-κB p65 were measured by western-blot. #p<0.05 *vs* before transfected (BT) or scramble transfected (ST). B-D. Protein expressions and relative grayscale ratios of TNF-α and IL-6 under 21% O_2 and 2% O_2 with or without 2.5 μg/ml LMWH in normal, TLR-4 siRNA transfected or NF-κB p65 siRNA transfected JEG-3 cells respectively. #p<0.05 *vs* 21% O_2, *p<0.05, **p<0.01 *vs* 2% O_2 and none LMWH.

In complex network of the synthesis and secretion of inflammatory factors, TLR-4 has been shown to be crucial for expression of various inflammatory cytokines. Binding of TLR-4 with its corresponding ligand, for example LPS, induces phosphorylation and degradation of IκBα, which in turn allows active NF-κB to translocate to nucleus and induce proinflammatory cytokine gene expression [33]. TLR-4 is widely expressed in interstitial trophoblast within pla-

cental bed of preeclamptic patients. Bernardi FC et al. observed a significant increase of protein levels of TLR-4 and NF-κB in placenta, and an increase of IL-6 levels in both plasma and placenta was reported [34]. Kim YM et al. also found TLR-4 protein expression was increased in interstitial trophoblasts of patients with PE, and they proposed that "danger signals" at feto-maternal interface, which were recognized by trophoblasts through TLR-4, may play a key role in creation of a local abnormal cytokine milieu [35]. In our *in vitro* experiments in JEG-3 cells, it was suggested that the anti-inflammatory effects of LMWH were due to the downregulation of nuclear factor-κB (NF-κB) signaling via TLR-4.

Multiple experiments have confirmed heparin can inhibit inflammatory response through TLR-4-NF-κB signaling [36, 37]. A study by Hasan Babazada et al. showed glycol-split heparin nanoparticles suppressed production of TNF-α in LPS-stimulated macrophages by inhibiting TLR4-mediated NF-κB signaling [17]. Another study from same authors suggested that selective inhibition of TLR-4-NF-κB signaling with lipid-modified heparin derivatives composited to nanostructures could provide an effective therapeutic approach to inhibit chronic inflammation in an animal model of rheumatoid arthritis [38]. However, anti-inflammatory mechanism of LMWH has not been extensively studied. Hence, the hypothesis of this study was LMWH also exerts its anti-inflammatory effects via TLR-4-NF-κB p65 signaling. Results showed LMWH significantly decrease mRNA and protein expressions of TNF-α and IL-6. However, this anti-inflammatory response was attenuated when dominant negative mutants for TLR-4/NF-κB p65 were used, suggesting the acting pathway of LMWH was TLR-4-NF-κB p65 signaling transportation.

In summary, we found LMWH could inhibit inflammatory response in placenta villous explants and JEG-3 cells by reducing expressions of TNF-α and IL-6, and the effects were attenuated after TLR-4 or NF-κB p65 blocking, further highlighting the concept of LMWH as an anti-inflammatory agent, in addition to its classical anticoagulatory properties. Other studies have shown that LMWH may also play a part in protecting vascular endothelial cells and promoting trophoblast cell invasion and anti-apoptosis [39].

In future research work, we attempt to further study signaling transfer mechanism of LMWH and apply LMWH to the clinical trial due to its potential for wider application in therapeutic strategies for women suffering from early-onset preeclampsia. This research may provide a new angle to understand the mechanism of severe PE treatment with LMWH.

Disclosure of conflict of interest

None.

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