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Effect of glutamine on cell adhesion molecule expression and leukocyte transmigration in endothelial cells stimulated by preeclamptic plasma

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Abstract

Objective: This study analyzed plasma glutamine (GLN) concentrations in women with preeclampsia. Also, in an in vitro study we evaluated whether GLN concentration was related to surface molecule expressions on endothelial cells (ECs) and polymorphonuclear neutrophils (PMNs) and the transendothelial migration of PMNs through ECs stimulated by preeclamptic plasma.

Methods: Blood samples were collected from 20 women with preeclampsia and 15 normal pregnant women for plasma GLN analysis. In the in vitro study, human umbilical vein endothelial cells and PMNs were treated with different concentrations (0, 300, 500, and 1000 μM) of GLN for 24 h. After that, we stimulated human umbilical vein endothelial cells for 3 h with plasma from patients with preeclampsia, and PMNs were allowed to transmigrate through ECs for 2 h. EC surface expressions of cellular adhesion molecules (CAMs) and integrin (CD11b) interleukin-8 (IL-8) receptor expressions on PMNs were measured by flow cytometry. The transendothelial migration of PMNs through ECs was also analyzed.

Results: Women with preeclampsia exhibited significantly lower plasma GLN concentrations than did normal pregnant women. The in vitro study showed that, compared with normal plasma, CAM expressions on human umbilical vein endothelial cells and PMNs were increased when preeclamptic plasma was stimulated. Among the groups with preeclamptic plasma stimulation, intracellular CAM-1 expression on ECs and CD11b and IL-8 receptor expressions on PMNs were lower with 500 and 1000 μM than with 300 μM of GLN. IL-8 production from ECs and PMNs was also lower with 500 and 1000 μM than with 300 μM of GLN. PMN transmigration was significantly higher with 300 μM of GLN than with the other GLN concentrations.

Conclusions: Plasma GLN is depleted in women with preeclampsia. The result of this in vitro study showed that ECs and PMNs were activated after preeclamptic plasma stimulation. A low GLN concentration resulted in greater CAM expression and greater transendothelial migration of neutrophils. GLN administration at levels similar to or higher than physiologic concentrations decreased IL-8 and CAM expressions, and PMN transmigration decreased after stimulation with preeclamptic plasma.

Keywords: Glutamine; Preeclampsia; Interleukin-8; Cellular adhesion molecules; Polymorphonuclear neutrophils transmigration

Introduction

Preeclampsia is a pregnancy-specific syndrome defined by the development of hypertension and proteinuria and/or pathologic edema appearing after 20 wk of gestation [1]. It is one of the leading causes of maternal and perinatal morbidity and mortality in developing countries. Recent studies have suggested that the basic pathophysiologic abnormality of preeclampsia is endothelial dysfunction accompanied by upregulated inflammation and immune processes [2,3]. The mechanisms leading to this dysfunction have not been clarified. Previous studies have found that cellular adhesion molecules (CAMs) are increased in the sera of patients with preeclampsia and that soluble CAM levels are associated

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with disease severity [4,5]. Adhesion molecules play a key role in cell-to-cell interactions and cell-to-extracellular matrix interactions. Immunoglobin gene superfamily intracellular adhesion molecule-1 (ICAM-1) and molecule-2 (ICAM-2) and vascular cell adhesion molecule-1 (VCAM-1) are expressed on activated endothelium [6–8]. ICAM-1 and VCAM-1 are important in the adhesion of monocytes, lymphocytes, and neutrophils to activated endothelium [6,7]. Previous reports have shown that the expression of ICAM-1 and VCAM-1 in human umbilical endothelial cells (HUVECs) is induced by plasma from women with preeclampsia [8,9].

Activated leukocytes express surface glycoproteins known as integrins, of which β_2 integrins (CD18) are particularly important [10]. CD11b/CD18 is abundant in neutrophils and contributes to neutrophil migration into sites of inflammation [11,12]. Migration of leukocytes into tissue is part of the host response in protecting an organ against tissue damage, but may also be harmful and may play a role in the pathogenesis of an inflammatory response [13,14].

Glutamine (GLN) is the most abundant free amino acid in the circulatory system. It serves as an important energy source for many rapidly proliferating cells, especially enterocytes and immune cells [15,16]. GLN deficiency can occur during periods of critical illness. In patients with catabolic diseases, plasma and muscle GLN levels are dramatically decreased, which correlates with a poor prognosis and the degree of protein catabolism in those patients [17,18]. GLN is considered to be an essential amino acid during certain inflammatory conditions and GLN supplementation is suggested in catabolic conditions [18,19]. A study by Fukatsu et al. [20] showed that, compared with conventional total parenteral nutrition, GLN-supplemented parenteral nutrition decreased ICAM-1 expression in intestinal homogenates. In addition, Arndt et al. [21] demonstrated that GLN administration decreases leukocyte adhesion and transmigration in indomethacin-induced intestinal inflammation in the rat. To the best of our knowledge, there has been no study that investigated the effect of GLN on the expression of CAMs and leukocyte transmigration in preeclampsia. We hypothesized that chronic inflammation during preeclampsia results in depletion of plasma GLN, and that GLN administration comparable to physiologic concentrations decreases CAM expressions in HUVECs induced by plasma from preeclamptic women and decreases migration of polymorphonuclear neutrophils (PMNs).

Materials and methods

Patients and sample information

The pregnant women were recruited from the Taipei Medical University/Municipal Wan Fang Hospital. Women with singleton pregnancies and no chronic diseases were included in this study. A normal pregnancy ($n = 15$) was one in which the women remained normotensive and nonproteinuric, women delivered at 37 to 42 wk, and the pregnancy was not complicated by fetal growth retardation or other fetal or maternal problems. Preeclampsia ($n = 20$) was diagnosed as a blood pressure of at least 140/90 mmHg on at least two occasions after the 20th week of gestation accompanied by 1+ proteinuria detected on a reagent strip on two occasions more than 4 h apart [4]. Mean gestational ages were 38.1 ± 1.5 wk (range 36~41) for the controls and 37.3 ± 1.9 wk (range 34~40) for women with preeclampsia. Among the 20 patients with preeclampsia, five were classified as having severe preeclampsia. Severe preeclampsia was defined as a blood pressure higher than 160/110 mmHg on two occasions 6 h apart and proteinuria corresponding to greater than 2+ detected on a reagent strip on two occasions more than 4 h apart [4]. We collected plasma samples from the five women who had severe preeclampsia for an in vitro study. The protocol was approved by the Wan-Fang Hospital ethics committee, and all subjects provided written informed consent before participation.

Plasma amino acid analysis

Blood samples were taken from control and preeclamptic patients at the time they came to the clinic for obstetric care, i.e., at 34 to 36 wk of gestational age. Antecubital venous blood from fasting women was drawn into tubes containing ethylene-diaminetetra-acetic acid. Blood was centrifuged at 300*g* for 10 min at 4°C, and plasma was stored at −70°C until being assayed. Plasma amino acids were analyzed by standard ninhydrin technology (model 6300, Beckman Instruments, Palo Alto, CA, USA) after deproteinization of plasma with 5% salicylic acid [22].

HUVEC isolation and culture

HUVECs were isolated from the umbilical cord vein according to the method of Jaffe et al. [23]. The umbilical vein was cannulated, washed with phosphate buffered saline (PBS), and perfused with PBS containing 0.1% collagenase for 10 min at 37°C in 5% CO_2. HUVECs were collected and established as a primary culture in medium-199 (M-199) containing 20% fetal bovine serum (FBS), 20 mM of $NaHCO_3$, 25 mM of HEPES, antibiotics (100 U/mL of penicillin and 100 μg/mL of streptomycin), 10 IU/mL of heparin sodium, and 15 mg/L of endothelial cell (EC) growth factor at 37°C in 5% CO_2 and 95% humidity. Cells were serially passaged approximately two to three times for the experimental assay.

Polymorphonuclear neutrophils

Venous blood was drawn from a 30-y-old healthy pregnant woman into heparinized tubes. A buffy coat, diluted three-fold with PBS, was applied on a Ficoll-Hypaque gradient and centrifuged for 30 min at 300*g* at 4°C. PMNs were

then isolated by Ficoll-Hypaque gradient centrifugation, with a density of 1.077 for 30 min at 300*g*. Neutrophils (95 ± 2% by May-Grünwald-Giemsa panoptic staining) obtained from the pellet of the Ficoll gradient were washed twice with PBS and resuspended in M-199 supplemented with 1% heat-inactivated FBS for 24 h to stabilize the PMNs, after which they were incubated with different concentrations of GLN (0, 300, 500, and 1000 μM) for 24 h (without FBS). Cells were 95 ± 2% viable as determined by May-Grünwald-Giemsa panoptic staining.

PMN migration across the endothelial monolayer

HUVECs (1×10^5 cells/well) from second subcultures were grown on fibronectin-coated inserts (3-μm pore size, 6.4 mm, Becton Dickson) which were then placed in a 24-well plate until the monolayer was confluent. They were then incubated in M-199 (without FBS) with different concentrations of GLN (0, 300, 500, and 1000 μM) for 24 h. The viability of HUVECs after incubation with different GLN concentrations was greater than 95% as determined by 0.2% trypan blue staining. Subsequently, cells were washed twice with PBS and cultured with various concentrations of GLN (without FBS), and 100 μL of plasma from normal or severely preeclamptic women was added in a final volume of 1 mL for 3 h. After that, PMNs (1×10^5 cells/100 μL/well) were added to the wells and allowed to migrate across the ECs for 2 h. Migration and descent of PMNs into the lower chamber was quantified by a microscopic counter in a hemocytometer [24].

Measurements of CAMs and interleukin-8 receptor expressions on PMNs and HUVECs

After the PMN-HUVEC interaction had proceeded for 2 h, HUVEC surface expressions of ICAM-1 and VCAM-1 and PMN expressions of CD11b and interleukin-8 (IL-8) receptor were measured. Solutions in the upper chambers of the transwells were collected and centrifuged at 1200 rpm for 10 min, and pellets were suspended in 100 μL of PBS for PMN analysis. After removing the supernatant, HUVECs were washed twice with PBS containing 2 mM of iced ethylene-diaminetetra-acetic acid to detach adherent PMNs, and then pellets were incubated with 100 μL of M-199 (without FBS and with 2 mM of iced ethylene-diaminetetra-acetic acid) for another 30 min at 4°C with the addition of fluorescein-conjugated mouse anti-human VCAM-1 (CD 106) and phycoerythrin-conjugated mouse anti-human ICAM-1 (CD 54). The suspension was collected into a tube and resuspended in 500 μL of PBS (containing 0.3 mL of 350 mM formaldehyde). The fluorescence intensity of a 5000-cell population was counted and analyzed by flow cytometry (Coulter, Miami, FL, USA). To determine integrin and IL-8 receptor expressions on PMNs, fluorescein-conjugated mouse anti-human CD11b (Serotec, Oxford, UK) and phycoerythrin-conjugated mouse anti-human CDw128a (Serotec) were added to 100 μL of the PMN suspension. Fluorescence data were collected on 1×10^5 viable neutrophils and analyzed by flow cytometry (Coulter).

Measurements of IL-8 and nitrite secreted by HUVECs and PMNs

Solutions in the upper chambers of the transwells were collected to determine IL-8 and nitric oxide (NO) secretions by ECs and neutrophils. IL-8 concentrations were measured using commercial enzyme-linked immunosorbent assay microtiter plates, with antibodies specific for human IL-8 having been coated onto the wells of the microtiter strips provided (Amersham Pharmacia Biotech, Buckinghamshire, UK). NO is highly unstable in solution and cannot readily be assayed. However, NO is converted to stable nitrite and nitrate ions in an aqueous solution. After conversion of nitrate to nitrite using nitrate reductase, nitrite concentrations were measured with the Griess reagent. Concentrations of NO_2^-/NO_3^- in the supernatant were determined with a commercial kit (Assay Designs, Ann Arbor, MI, USA). Procedures followed the manufacturer's instructions.

Statistical analysis

Data are expressed as means ± standard deviations. Results are representative of three independent experiments. Differences across groups were analyzed by analysis of variance using Duncan's test. $P < 0.05$ was considered statistically significant.

Results

Plasma GLN levels

Plasma GLN levels in the preeclampsia group were significantly lower than those in the normal pregnant group (349.8 ± 55.1 versus 498.3 ± 47.4 μmol/L, $P < 0.05$).

CAM expressions on HUVECs

ICAM-1 and VCAM-1 expressions on HUVECs were higher when stimulated with preeclamptic plasma than with normal plasma. Among the groups stimulated with preeclamptic plasma, ICAM-1 expressions on HUVECs were higher with 300 μM of GLN than with 0, 500, and 1000 μM of GLN, and expression with 1000 μM of GLN was even lower than that with 500 μM of GLN. There were no differences in VCAM-1 expression among samples incubated with 300, 500, and 1000 μM of GLN (Table 1).

Table 1
ICAM-1 and VCAM-1 expressions on endothelial cells stimulated with normal and preeclamptic plasma at various GLN concentrations*

GLN	ICAM-1 (%)	VCAM-1 (%)
0 μM		
Normal	3.51 ± 0.92†	25.92 ± 2.33
Preeclampsia	9.62 ± 0.93‡	27.34 ± 2.32‡
300 μM		
Normal	3.04 ± 1.26†	24.31 ± 1.22†
Preeclampsia	14.97 ± 0.55‡	44.44 ± 2.43
500 μM		
Normal	3.31 ± 1.22†	23.65 ± 1.12†
Preeclampsia	13.47 ± 0.33‡	45.70 ± 2.42
1000 μM		
Normal	3.71 ± 0.92†	22.83 ± 3.10†
Preeclampsia	11.44 ± 0.46	46.65 ± 1.76

GLN, glutamine; ICAM-1, intracellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1.

* Data are presented as mean ± standard deviation. Results are representative of three independent experiments.

† Significantly lower than the preeclampsia group at the same GLN concentration.

‡ Significantly different from other GLN concentrations in the preeclampsia group.

CD11b and IL-8 receptor expressions on PMNs

CD11b and IL-8 receptor expressions on PMNs were higher when stimulated with preeclamptic plasma than with normal plasma. Among the groups stimulated with preeclamptic plasma, CD11b and IL-8 receptor expressions on PMNs were higher with 300 μM of GLN than with 500 or 1000 μM of GLN. There were no differences in CD11b and IL-8 receptor expressions between 500 and 1000 μM of GLN (Table 2).

IL-8 and NO_2^-/NO_3^- released by HUVECs and PMNs

IL-8 production by ECs and neutrophils was higher when stimulated with preeclamptic plasma and GLN supplementation than with normal plasma, whereas no differences in NO_2^-/NO_3^- concentrations were observed between normal and preeclampsia plasmas at various GLN concentrations. IL-8 levels were significantly lower with 500 or 1000 μM than with 300 μM of GLN, and there were no differences in IL-8 levels between 500 and 1000 μM of GLN when stimulated with preeclamptic plasma (Table 3).

PMN migration across the endothelial monolayer

There were no differences in PMN migration across the endothelial monolayer among various GLN concentrations in the normal group. PMN transmigration was significantly higher in the preeclamptic group with 300 μM of GLN than with other GLN concentrations (Fig. 1).

Table 2
CD11b and IL-8 receptor expressions on neutrophils after incubation with various GLN concentrations and stimulation with plasma from normal and preeclampsia*

GLN	IL-8 receptor (%)	CD11b (%)
0 μM		
Normal	3.50 ± 0.51†	15.57 ± 1.25†
Preeclampsia	7.28 ± 0.46‡	34.60 ± 5.65‡
300 μM		
Normal	4.15 ± 0.94†	17.54 ± 1.95†
Preeclampsia	16.01 ± 0.12‡	59.64 ± 2.92‡
500 μM		
Normal	4.55 ± 0.64†	16.52 ± 0.91†
Preeclampsia	14.41 ± 0.42	40.66 ± 4.94
1000 μM		
Normal	3.31 ± 0.71†	15.15 ± 0.84†
Preeclampsia	14.09 ± 0.85	39.60 ± 2.12

GLN, glutamine; IL-8, interleukin-8.

* Data are presented as mean ± standard deviation. Results are representative of three independent experiments.

† Significantly lower than the preeclampsia group at the same GLN concentration.

‡ Significantly different from other GLN concentrations in the preeclampsia group.

Discussion

This study is the first to report that plasma from women with preeclampsia had significantly lower GLN levels than did plasma from normal pregnant women. This finding is compatible with previous reports that plasma GLN is decreased during catabolic conditions such as inflammation, infection, and injury [17,18,25]. To understand whether GLN concentrations may have an effect on CAM expres-

Table 3
IL-8 and NO_2^-/NO_3^- secretions by endothelial cells and neutrophils stimulated by normal or preeclamptic plasma at different GLN concentrations*

GLN	IL-8 (pg/mL)	NO_2^-/NO_3^- (μM)
0 μM		
Normal	33.23 ± 0.81	34.80 ± 2.52
Preeclampsia	32.22 ± 1.14‡	33.27 ± 2.65
300 μM		
Normal	34.57 ± 0.75†	33.44 ± 1.95
Preeclampsia	50.30 ± 0.82‡	32.21 ± 2.52
500 μM		
Normal	34.11 ± 0.93†	30.03 ± 2.27
Preeclampsia	42.15 ± 1.28	30.55 ± 1.38
1000 μM		
Normal	33.57 ± 1.36†	29.84 ± 3.22
Preeclampsia	40.72 ± 1.51	30.10 ± 1.13

GLN, glutamine; IL-8, interleukin-8; NO, nitric oxide

* Data are presented as mean ± standard deviation. Results are representative of three independent experiments.

† Significantly lower than the preeclampsia group at the same GLN concentration.

‡ Significant difference from other GLN concentrations in the preeclampsia group.

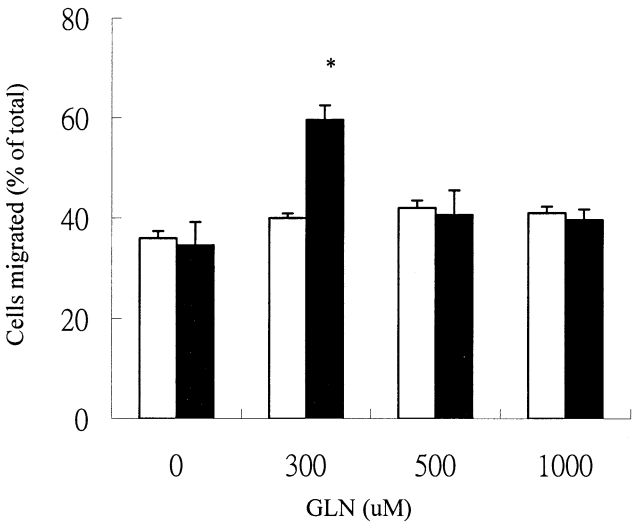

Fig. 1. Normal plasma (white bars) and preeclamptic plasma (black bars) stimulated migration of polymorphonuclear neutrophils across human umbilical vein endothelial cells cultured on a human fibronectin-coated culture with the addition of different GLN concentrations. Results are representative of three independent experiments. *Polymorphonuclear neutrophils across endothelial cells with 300 μM in the preeclampsia group were significantly higher than those with 0, 500, and 1000 μM of GLN. No difference in polymorphonuclear neutrophils across endothelial cells in the normal group was observed at various GLN concentrations. GLN, glutamine.

sion in preeclampsia, we treated ECs and PMNs with different GLN concentrations including low (300 μM), approximately physiologic (500 μM), and high (1000 μM) in an in vitro study after stimulation of preeclamptic plasma. Although ECs were not obtained from patients with preeclampsia, it seems that ECs were activated in this model, because we observed an increase in CAM expression on HUVECs and PMNs after stimulation with preeclamptic plasma compared with normal plasma.

Endothelial activation and dysfunction are central pathogenic features in women with preeclampsia, which is a multiple-system disorder during human pregnancy [2]. It has been suggested that preeclampsia represents an excessive maternal inflammatory response to pregnancy [26]. We observed that adhesion molecules (CD11b, ICAM-1, and VCAM-1) and IL-8 receptor expression on HUVECs and neutrophils were lowest in the preeclamptic groups without GLN than with GLN administration. The preeclamptic group with 300 μM of GLN had higher expression of adhesion molecules than did the corresponding groups with 0, 500, and 1000 μM of GLN. Glucose is the primary fuel for leukocytes and rapidly proliferating cells; however, the rate of GLN utilization by these cells is similar to or greater than that of glucose [27,28]. In the absence of GLN, glucose may be used as an energy source for maintaining basal function of cells; whenever GLN is available for use, adhesion molecules are expressed in response to stimulation of preeclamptic plasma. ICAM-1 is the ligand counterpart of integrins on PMNs. CD11b is the most abundant integrin on PMNs and has a major role in neutrophil adhesion and diapedesis. PMN-to-EC adhesion involves ICAM-1 and CD11b, which allow migration of neutrophils across the vascular endothelium, where final tissue destruction occurs. In this study, we found that GLN administration at levels similar to or higher than physiologic concentrations decreased ICAM-1 expression on vascular ECs and CD11b on neutrophils as compared with the low GLN group. Fukatsu et al. [21] reported that GLN-supplemented parenteral nutrition decreased gut ICAM-1 expression in rats, possibly by recovery of decreased intestinal interleukin-4 production. Because T-helper type 2 cytokines are secreted by T lymphocytes, mechanisms other than modulating interleukin-4 might be responsible for the lower CAM expression in the high GLN group observed in this study. A previous study showed that plasma from women with preeclampsia upregulate HUVEC nuclear factor-κB activity and ICAM-1 expression compared with plasma from normal pregnant women [9]. Whether nuclear factor-κB is involved in GLN-mediated decreased CAM expression is under investigation in our laboratory.

NO is a product of the enzymatic conversion of arginine to citrulline, a major messenger involved in the control of inflammation, and an important regulator of placental perfusion [29]. Previous reports have shown that a dysfunction of the L-arginine–NO pathway is the characteristic of preeclampsia. An in vitro study revealed that mRNA and protein expressions for endothelial NO synthase were decreased in ECs from preeclamptic pregnancies compared with cells from control pregnancies [30]. A study by Benedetto et al. [31] showed that NO production is enhanced in severe preeclampsia, possibly as a compensatory phenomenon for the increased synthesis and release of vasoconstrictors and platelet aggregating agents. Because GLN was shown to modulate NO synthesis in cultured ECs [32,33], assessing the effect of GLN on NO production as an index of NOS activity seems relevant. In this study there were no differences in nitrite concentrations among preeclamptic groups with various GLN levels. This finding indicated that the influence of GLN on NO production was not obvious. However, this result was consistent with an in vitro study by Coeffier et al. [34] who also found that nitrite production is not affected by GLN in the human gut.

IL-8 is a potent neutrophil chemoattractant and activator secreted by ECs and leukocytes. IL-8 initiates the acute inflammatory cascade and is an early marker of the inflammatory process. A previous report showed that an increase in IL-8 results in increased endothelial permeability in preeclampsia [30]. In this study, we found that IL-8 secretion with 500 and 1000 μM was significantly lower than that with 300 μM of GLN when stimulated with preeclamptic plasma. This finding parallels that of the effects of GLN on PMN IL-8 receptor expression. This result suggests that a lower GLN concentration results in higher IL-8 expression, whereas nearly normal or higher than physiologic GLN decreases IL-8 production stimulated by preeclamptic

plasma. An in vitro study by Huang et al. [35] showed that GLN-decreased lipopolysaccharide-induced IL-8 production in Caco-2 cells is not associated with nuclear factor-κB binding. Coeffier et al. [34] reported that a high GLN concentration had inhibitory effects on IL-8 mRNA and protein expressions. Determining the mechanisms by which GLN decreases preeclampsia-induced IL-8 production in HUVECs requires further investigation.

In this study, we used monolayers of cultured ECs as a barrier to investigate the effects of GLN on leukocyte migration through the endothelium under stimulation by preeclamptic plasma. We measured only the transmigration of neutrophils because PMNs are the largest population of leukocytes. Galdiero et al. [36] used bacterial products as stimulants to investigate the effects of different inducers on transendothelial migration of leukocytes. They found that, among leukocyte populations, transmigration of neutrophils was most obvious. In this study, we found that transmigration of PMNs was significantly lower with physiologic levels of GLN than with lower levels of GLN. We speculated that GLN administration at levels similar to physiologic concentrations decreases IL-8 and CAM expressions, which may consequently result in a lower extent of PMN–HUVEC interactions and PMN migration.

In summary, this study has demonstrated that plasma from women with preeclampsia has significantly lower GLN levels than that from normal pregnant women. The in vitro study showed that HUVECs and PMNs are activated after stimulation with preeclamptic plasma. A low GLN concentration resulted in higher adhesion molecule expressions and greater transendothelial migration of neutrophils. GLN administration at levels similar to or higher than physiologic concentrations decreased IL-8 and adhesion molecule expressions on ECs and neutrophils; PMN transmigration also decreased under stimulation with preeclamptic plasma. These results provide an additional rationale for evaluating GLN supplementation in patients with preeclampsia.

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