Low Molecular Weight Heparin Improves Endothelial Function in Pregnant Women at High Risk of Preeclampsia

Kelsey McLaughlin, Dora Baczyk, Audrey Potts, Michelle Hladunewich, John D. Parker, John C.P. Kingdom

Abstract—Low molecular weight heparin (LMWH) has been investigated for the prevention of severe preeclampsia, although the mechanisms of action are unknown. The objective of this study was to investigate the cardiovascular effects of LMWH in pregnant women at high risk of preeclampsia. Pregnant women at high risk of preeclampsia (n=25) and low-risk pregnant controls (n=20) at 22 to 26 weeks' gestation underwent baseline cardiovascular assessments. High-risk women were then randomized to LMWH or saline placebo (30 mg IV bolus and 1 mg/kg subcutaneous dose). Cardiovascular function was assessed 1 and 3 hours post randomization. The in vitro endothelial effects of patient serum and exogenous LMWH on human umbilical venous endothelial cells were determined. High-risk women demonstrated a reduced cardiac output, high resistance hemodynamic profile with impaired radial artery flow-mediated dilation compared with controls. LMWH increased flow-mediated dilation in high-risk women 3 hours after randomization compared with baseline and increased plasma levels of placental growth factor, soluble fms-like tyrosine kinase-1, and myeloperoxidase. Serum from high-risk women impaired endothelial cell angiogenesis and increased PlGF-1 and PlGF-2 transcription compared with serum from low-risk controls. Coexposure of high-risk serum with LMWH improved the in vitro angiogenic response such that it was equivalent to that of low-risk serum and promoted placental growth factor secretion. LMWH improves maternal endothelial function in pregnant women at high risk of developing preeclampsia, possibly mediated through increased placental growth factor bioavailability. (Hypertension. 2017;69:180-188. DOI: 10.1161/HYPERTENSIONAHA.116.08298) • Online Data Supplement

Key Words: endothelium • heparin • PlGF • preeclampsia • pregnancy

Preeclampsia is a hypertensive disorder of pregnancy characterized by new-onset hypertension with evidence of organ injury, affecting 2% to 8% of all pregnancies worldwide.1 The majority of women with preeclampsia present clinically near term with favorable maternal and infant outcomes; however, a subset of women develop severe disease characterized by the need for preterm delivery typically before 34 weeks of gestation because of end-organ injury, severe hypertension, or intruterine growth restriction.2 Women with severe preeclampsia demonstrate significant cardiovascular impairment during pregnancy and the immediate postpartum period and a higher risk of cardiovascular disease later in life.3,4 Given the significant short- and long-term maternal cardiovascular effects of preeclampsia, elucidating its pathogenesis has been of much interest in recent years. However, as yet, there is no agreement concerning the most effective pharmacological therapy for the prevention of preeclampsia in screen-positive women beyond aspirin, with clinical symptoms typically only resolving on delivery.8

Low molecular weight heparin (LMWH) has been evaluated for the prevention of various placenta-mediated pregnancy complications, including severe preeclampsia and recurrent miscarriage. Multiple trials and systematic reviews have concluded that LMWH reduces the incidence of recurrent severe preeclampsia in high-risk women, as well as perinatal mortality, preterm birth, and infant birth weight <10th percentile for gestational age, whereas other studies have found no treatment effect.9,10 Because the majority of previous trials have consisted of clinical end points, the mechanism of action of LMWH for the possible prevention of severe preeclampsia is currently unknown. Evidence suggests that observed beneficial effects do not result from heparin’s anticoagulant actions within the placenta.11,12 An alternative hypothesis is that LMWH exerts vascular actions in the maternal compartment that reverse the systemic vascular dysfunction characteristic of preeclampsia. Previous trials in patients with coronary artery disease have determined that LMWH demonstrates beneficial endothelial effects, possibly through increased bioavailability of NO.13,14

The objective of the current study was to investigate the endothelial effects of LMWH in pregnant women at high risk of preeclampsia. We first compared baseline cardiovascular...
function in pregnant women at high risk of severe preeclampsia with healthy pregnant controls. The acute in vivo endothelial effects of LMWH were then evaluated in high-risk pregnant women followed by the in vitro endothelial effects of LMWH, coexposed with patient serum, using angiogenesis assays, gene expression, and protein secretion methods.

Methods

Selection of Low- and High-Risk Pregnant Subjects
Healthy pregnant women with no clinical risk factors were recruited from the Prenatal Clinics at Mount Sinai Hospital to act as a low-risk control group. Inclusion criteria of low-risk women included women with a previable singleton pregnancy at 22 to 26 weeks’ gestation with no maternal or fetal health concerns. Pregnant women at high risk of developing preeclampsia were recruited from the Maternal-Fetal Medicine Division, Placenta Clinic at Mount Sinai Hospital, which provides antenatal care to pregnant women at risk of developing placental complications, including preeclampsia and intrauterine growth restriction. Inclusion criteria included previable singleton pregnancy at 22 to 26 weeks’ gestation accompanied by at least 2 of the following inclusion categories predicted to increase the risk of developing placenta-mediated complications of pregnancy: (1) abnormal placental biochemistry; (2) abnormal placental shape or texture; (3) abnormal uterine artery Doppler; and (4) abnormal clinical risk factor score. This inclusion criteria are described in detail in the Methods section in the online-only Data Supplement. Eligible subjects were invited to participate in the study and provided written informed consent. This study was reviewed and approved by the Human Subjects Review Committee of Mount Sinai Hospital (MSH REB 12-0083-A).

Study Protocol
Study visits were conducted in a quiet temperature- and humidity-controlled environment. Subjects were required to abstain from caffeine on the study day and consume a light breakfast. Participants came to the laboratory at 8.00 AM. After 15 minutes of rest, baseline blood pressure and heart rate were recorded in the left arm with subjects in the sitting position using the automatic, calibrated sphygmomanometer Dinamap Pro 400 (GE Healthcare). Flow-mediated dilation (FMD) and flow-mediated constriction of the radial artery was then performed (Vivid 7; GE Healthcare, Canada; M12L). Noninvasive measures of cardiac output were used to assess maternal hemodynamics (Cheetah Medical, Vancouver, WA). The pulsatility index of the uterine arteries was determined using pulsed-wave Doppler (Philips Medical Systems, Eindhoven, the Netherlands). Urine and venous blood samples were collected and stored. After this baseline assessment, low-risk subjects were discharged from the laboratory. High-risk subjects were then randomized by a designated study nurse of the Mount Sinai Clinical Cardiovascular Research Laboratory to the placebo (saline) or LMWH (enoxaparin) treatment group in a double-blind manner. Subjects received an intravenous bolus (30 mg) followed by a subcutaneous dose (1 mg/kg) of their respective agent. High-risk subjects repeated the assessment as described above 1 and 3 hours after randomization.

Experimental Procedures
Radial artery diameter was continuously recorded throughout a baseline period (5 minutes), during wrist cuff inflation (250 mmHg for 4.5 minutes) and after wrist cuff deflation (4.5 minutes). FMD was calculated as the maximum percent change in radial artery diameter after wrist cuff release compared with baseline radial artery diameter. Flow-mediated constriction was calculated as the maximum percent change in radial artery diameter during wrist cuff inflation compared with baseline radial artery diameter. Analysis was performed in a blinded fashion using automatic custom-designed vascular edge detection software. Hemodynamic monitoring was performed for 15 minutes at rest with the noninvasive measures of cardiac output system (Cheetah Medical), using transthoracic bioreactance technology to estimate stroke volume, with sensor ECG detection of heart rate. Detailed methods are provided in the Methods section in the online-only Data Supplement.

Plasma and Urine Protein Immunoassays and Clotting Time
Venous blood samples were collected from the antecubital vein by single venipuncture in the low-risk group and serially through a 5F cannula in the high-risk group, spun, and stored at −80 °C. Plasma samples were quantified using ELISA for placental growth factor (PIGF), soluble fms-like tyrosine kinase-1 (sFlt-1), soluble endoglin, myeloperoxidase (MPO), endothelin, and vascular endothelial growth factor (R&D Systems, Burlington, Ontario, Canada). The activated partial thromboplastin time assay was used to determine plasma clotting time (Pacific Hemostasis; Thermo Scientific, Hudson, NH). Urine samples were collected whole and stored at −80 °C and quantified for PIGF (R&D Systems). All ELISAs were performed according to manufacturers’ instructions. Detailed methods are provided in the Methods section in the online-only Data Supplement.

In Vitro Endothelial Cell Functional Assays
The effects of subject serum and LMWH treatments on angiogenesis were assessed in human umbilical vein endothelial cells (HUVEC). Serum treatments were added to each well (low risk, n=6; high risk, n=5) as follows: (1) 5% fetal bovine serum (FBS) control, (2) 5% low-risk subject serum, (3) 5% low-risk subject serum and LMWH (2.5 U/mL), (4) 5% high-risk subject serum, and (5) 5% high-risk subject serum and LMWH (2.5 U/mL). The capillary-like structures formed by differentiating HUVECs were quantified through measuring the total tube length and number of branch points in each well in a manual manner blinded to clinical information. Data were presented as the average of duplicate samples and expressed as a percentage normalized to FBS control. The effects of subject serum and LMWH treatments on HUVEC proliferation and toxicity were assessed using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI) and CytoScan SRB Cytotoxicity Assay (G Biosciences, Maryland Heights, MO) according to the manufacturers’ instructions. Detailed methods are provided in the Methods section in the online-only Data Supplement.

In Vitro Endothelial Cell Gene Expression and Protein Levels
HUVEC cells were plated in 24-well plates and cultured for 24 hours in media containing subject serum (low risk, n=6; high risk, n=4) as follows: (1) 5% FBS control, (2) 5% low-risk subject serum, (3) 5% low-risk subject serum and LMWH (2.5 U/mL), (4) 5% high-risk subject serum, and (5) 5% high-risk subject serum and LMWH (2.5 U/mL). Total RNA was extracted. mRNA was reversed, and quantitative real-time PCR was performed to evaluate HUVEC gene expression of PIGF-1, PIGF-2, and sFlt-1. All data were normalized to housekeeping genes and expressed as a fold change relative to the FBS control. HUVEC cells were plated in 24-well plates and cultured for 24 hours in media containing subject serum (low risk, n=3; high risk, n=3), as follows: (1) 5% FBS control, (2) 5% low-risk subject serum, (3) 5% low-risk subject serum and LMWH (2.5 U/mL), (4) 5% high-risk subject serum, and (5) 5% high-risk subject serum and LMWH (2.5 U/mL). The cell lysates were quantified for PIGF (R&D Systems). De novo protein secretion was analyzed by quantifying PIGF, sFlt-1, and vascular endothelial growth factor levels in the media samples (R&D). Results were normalized to the levels of protein in the respective original serum treatment and presented as relative to FBS. Detailed methods are provided in the Methods section in the online-only Data Supplement.

Statistical Analysis
Statistical analyses were performed using SAS (version 9.4; SAS Institute, Cary, NC). Sample size estimates assumed 1−β=0.2 and a 2-sided α of 0.05. We were unable to identify previous studies that evaluated radial artery FMD in pregnant women at high risk of preeclampsia at 22 to 26 weeks’ gestation. Sample size estimates were made based on data from a previous report assessing
brachial artery FMD at 23 to 25 weeks' gestation, in which pregnant women who developed preeclampsia demonstrated impaired FMD (3.6±2.8%) compared with pregnant controls (8.6±2.8%), and a previous report from our laboratory assessing radial artery FMD in patients with hypertension (3.2±2.0%). Detection of a 30% difference in radial artery FMD in pregnant women at both low and high risk of preeclampsia at 80% power required a sample size of 20, for a total of 40 subjects. To detect a treatment effect causing a 50% change in radial artery FMD, we estimated that 12 high-risk women in each of the treatment arms would be reasonable and feasible, for a total high-risk sample size of 24. We, therefore, recruited 20 low-risk pregnant women and 26 high-risk women, to take a more conservative approach and account for potential dropouts.

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<th>Table. Demographic, Hemodynamic, and Delivery Characteristics of Pregnant Women at Low and High Risk of Preeclampsia</th>
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Data are presented as mean±SEM, median (interquartile range), or n (%) as appropriate. Composite adverse pregnancy outcome is defined as at least 1 of the following: stillbirth/neonatal death, IUGR, preeclampsia, gestational hypertension, or gestational age <37 wk. BMI indicates body mass index; C/S, cesarean section; IUGR, intrauterine growth restriction; and PI, pulsatility index.
Normality was assessed using the Shapiro–Wilk test. Baseline comparisons between pregnant women at low and high risk of preeclampsia were performed with an unpaired t test, χ² test, or Wilcoxon Mann–Whitney test. Paired within-group comparisons were performed with a paired t test or the Wilcoxon signed rank-sum test. Comparisons for multiple time points in the high-risk group were performed with repeated-measures ANOVA followed by the Bonferroni method for multiple comparisons of normally distributed data or the Friedman test for non-normally distributed data. Correlations were assessed by Pearson coefficients. Normally distributed data are presented as means±SEM, whereas non-normally distributed data are presented as median±interquartile range. P values of <0.05 were set as the threshold for significance.

Results

Demographics Characteristics and Pregnancy Outcomes of Pregnant Women at Low and High Risk of Preeclampsia

Twenty low-risk pregnant women and 26 high-risk pregnant women were recruited for study participation, with 1 high-risk subject subsequently withdrawing consent for personal reasons. There were no significant differences in maternal age, ethnicity, BMI, maternal comorbidity, or gestational age between pregnant women at low and high risk of preeclampsia (Table). High-risk women had a higher incidence of previous cesarean section (P=0.04) compared with low-risk women (Table). At the time of delivery, high-risk women demonstrated significantly lower gestational age (P<0.0001) and birth weight (P<0.0001) when compared with low-risk women, whereas incidence of adverse pregnancy outcome in high-risk women was significantly increased (P<0.0001; Table). Delivery information was not available for 1 low-risk subject and 1 high-risk subject.

Baseline Hemodynamic Characteristics and Endothelial Function

At 24 weeks' gestation, pregnant women at high risk of developing preeclampsia demonstrated significantly higher levels of systolic, diastolic, and mean arterial blood pressure and heart rate when compared with low-risk women (P<0.05; Table), although no women were clinically hypertensive. High-risk women had lower stroke volumes and cardiac outputs compared with low-risk women (P<0.0005; Table). In addition, total peripheral resistance and uterine artery pulsatility index were elevated in high-risk women (P<0.0005; Table). Baseline radial artery FMD was significantly lower in high-risk women compared with low-risk women (6.5±0.9% versus 9.7±1.0%; P=0.03; Figure 1), although there were no differences in radial
artery flow-mediated constriction. There were no differences between low and high risk women in radial artery diameter, blood flow, or reactive hyperemia (Table S3 in the online-only Data Supplement).

**Plasma and Urine Levels of Angiogenic Proteins**

At 24 weeks’ gestation, pregnant women at high risk of pre-eclampsia demonstrated significantly lower levels of plasma and urine PI GF compared with low-risk women (185 pg/mL [67–341] versus 444 pg/mL [256–540]; P=0.002; Figure S1A and 46 pg/mL [16–54] versus 153 [68–190]; P=0.003; Figure S1B, respectively). Soluble endoglin plasma levels were significantly higher in high-risk women compared with low-risk women (9 ng/mL [5–12] versus 6 ng/mL [5–7]; P=0.003; Figure S1C). No differences were detected between groups in plasma levels of sFlt-1, MPO, or endothelin-1 (Figure S1D through S1F, respectively). Plasma PI GF correlated negatively with total peripheral resistance and uterine artery pulsatility index (r=−0.61; P<0.0001; Figure S2A and r=−0.58; P<0.0005; Figure S2B, respectively).

**Hemodynamic Characteristics and Endothelial Function in Response to LMWH**

The plasma of high-risk women randomized to LMWH demonstrated significantly increased clotting time after infusion compared with baseline (24±8 seconds versus 13±4 seconds; P=0.03; Figure S3), whereas no changes in clotting time were detected in women randomized to placebo. High-risk women randomized to placebo or heparin did not demonstrate any significant differences in systolic, diastolic, or mean arterial blood pressure at 1 hour or 3 hours post randomization compared with baseline. Similarly, no significant changes in stroke volume, cardiac output, total peripheral resistance, or flow-mediated constriction were demonstrated in high-risk women post randomization to LMWH. In high-risk women randomized to LMWH, FMD was significantly increased 3 hours post randomization compared with baseline (10.8±1.0% versus 7.6±1.0%; P=0.008; Figure 2), whereas no differences were demonstrated in women randomized to placebo. There were no between or within randomization group differences in radial artery diameter, blood flow, or reactive hyperemia (Table S3).

**Plasma Angiogenic Proteins in Response to LMWH**

High-risk women randomized to placebo did not demonstrate any changes in plasma angiogenic proteins after randomization. After LMWH randomization, high-risk women demonstrated significant increases in plasma levels of PI GF (P=0.006; Figure 3A), sFlt-1 (P=0.03; Figure 3B), and MPO (P=0.03; Figure 3C). No changes in soluble endoglin or endothelin-1 were detected after randomization.

**Angiogenesis, Proliferation, and Toxicity Responses to Pregnant Serum and LMWH**

Representative images are provided demonstrating the HUVEC angiogenic effects of serum from the positive FBS control, pregnant women at low risk of preeclampsia, and pregnant women at high risk of preeclampsia (Figure 4A through 4C). Serum from pregnant women at high risk of preeclampsia significantly arrested HUVEC angiogenesis, compared with serum from low-risk pregnant women (0.79±0.05 versus 0.99±0.05; P=0.03; Figure 4D). The addition of LMWH to serum from high-risk women significantly increased angiogenesis, compared with serum from high-risk women alone (1.00±0.09 versus 0.79±0.05; P=0.02; Figure 4D), whereas no differences were demonstrated with serum from low-risk women. Proliferation and toxicity of HUVECs were not modified by serum from low- or high-risk pregnant women or LMWH.

![Figure 3. Plasma proteins of pregnant women at high risk of preeclampsia after low molecular weight heparin (LMWH) randomization. LMWH significantly increased plasma levels of placental growth factor (PI GF, A; P=0.006), soluble fms-like tyrosine kinase-1 (sFlt-1, B; P=0.03), and myeloperoxidase (MPO, C; P=0.03) in women at high risk of preeclampsia (n=13). No significant differences were detected in the placebo group. Data are normalized to each subjects’ baseline plasma levels (set as 1, dashed line) and presented as medians±25th and 75th percentile confidence intervals.](http://hyper.ahajournals.org/doi/fig/10.1161/HYPERTENSIONAHA.116.116592)
Gene Expression and Protein Secretion in Response to Serum of Pregnant Women and LMWH

Transcription of PlGF-1 and PlGF-2 was significantly increased in HUVECs exposed to the serum of pregnant women at high risk of preeclampsia, when compared with serum of low-risk women (1.71 [1.3–2.3] versus 0.44 [0.4–0.6]; P=0.03; Figure 5A and 1.76 [1.3–2.4] versus 0.55 [0.4–0.7]; P=0.01; Figure 5B, respectively). Coexposure of subject serum with LMWH did not modify PlGF-1 or PlGF-2 expression (Figure 5A and 5B, respectively). No differences in HUVEC mRNA expression of sFlt-1 were detected in exposure to low- or high-risk serum or with LMWH treatment.

HUVEC intracellular and media levels of PlGF did not significantly differ between cells exposed to serum from low-risk and high-risk pregnant women (Figure 5C and 5D, respectively). Coexposure of serum from low- and high-risk pregnant women with LMWH significantly decreased intracellular PlGF levels (2.1±0.4 pg/mL versus 1.3±0.3 pg/mL; P=0.02 and 1.6±0.2 pg/mL versus 1.2±0.2 pg/mL; P=0.004, respectively; Figure 5C) and significantly increased PlGF secretion into the media (1.0±0.2 pg/mL versus 2.3±0.3 pg/mL; P=0.005 and 0.6±0.1 pg/mL versus 2.1±0.4 pg/mL; P=0.02, respectively; Figure 5D). No changes in sFlt-1, MPO, or vascular endothelial growth factor secretion were detected with LMWH treatment.

Discussion

In the present study, we confirm that pregnant women at high risk of preeclampsia demonstrate significant cardiovascular abnormalities compared with low-risk women at 24 weeks’ gestation, supported by in vivo and in vitro findings. We provide the first human documentation that LMWH acutely modifies the endothelial function and circulating angiogenic proteins of pregnant women at high risk of preeclampsia. In parallel, in vitro endothelial function was also influenced by LMWH, with significant angiogenic responses and effects on PlGF secretion.

Our finding that pregnant women at high risk of preeclampsia demonstrate significant cardiovascular impairment before the clinical presentation of preeclampsia is consistent with previous reports. Although the high-risk group did not exhibit features of preeclampsia at the time of the study, they demonstrated a high resistance, low stroke volume profile with evidence of elevated blood pressure and endothelial dysfunction at 24 weeks’ gestation. Recognition of abnormal adaptations to pregnancy may allow for identification of women vulnerable to future cardiovascular disease, providing a window for potential therapeutic intervention.

At 24 weeks’ gestation, plasma levels of PlGF and soluble endoglin differed significantly between low- and high-risk pregnant women. Plasma PlGF levels were negatively correlated with total peripheral vascular resistance and uterine artery pulsatility index, suggesting a potential role of PlGF in mediating normal maternal vascular adaptation during pregnancy. These findings are consistent with recent reports that plasma PlGF is an accurate diagnostic tool for preeclampsia in pregnant women presenting with suspected disease and is now being evaluated real time in clinical practice in some centers.

To assess how these circulating proteins could impact endothelial cell function, we investigated the effects of low-risk and high-risk patient serum on in vitro endothelial cell angiogenesis, gene expression, and protein secretion. Serum from high-risk women significantly impaired angiogenesis and, interestingly, increased endothelial cell expression of...
both \textit{PlGF-1} and \textit{PlGF-2} compared with the serum of low-risk women, although these steady-state transcriptional changes were not reflected in either the intracellular or secreted levels of \textit{PlGF} into the culture media. Collectively, these results suggest a potential role for \textit{PlGF} in mediating endothelial function in pregnant women.

We demonstrate for the first time that LMWH acutely improves endothelium-dependent vascular function in pregnant women at high risk of preeclampsia. Furthermore, in vitro coexposure of serum of high-risk women with LMWH restored endothelial tube formation, consistent with previous reports from our group and others. These results illustrate a potential mechanism whereby LMWH could exert a beneficial effect on maternal endothelial function, as previously demonstrated in patients with coronary artery disease. These observations are consistent with a previous trial demonstrating that LMWH therapy throughout pregnancy prevented the rise of systolic and diastolic blood pressure and resistance of uterine arteries in pregnant women at high risk of preeclampsia, in addition to reducing the recurrence of preeclampsia. Interestingly, administration of LMWH to a rat model of preeclampsia reduced proteinuria and blood pressure. Previous ex vivo and in vitro work have determined that LMWH is an endothelium-dependent vasodilator, enhancing NO bioavailability in endothelial cells. Overall, our findings are consistent with previous human and animal trials, suggesting that LMWH exerts beneficial endothelial effects.

LMWH induced significant increases in circulating plasma levels of \textit{PlGF}, sFlt-1, and MPO in high-risk subjects. Heparin administration has previously been shown to substantially increase circulating levels of these proteins, in women with preeclampsia and other populations. In vitro, however, the administration of LMWH to serum samples significantly decreased intracellular levels of \textit{PlGF} in both groups with a concurrent increase in secreted \textit{PlGF} levels, whereas sFlt-1 and MPO levels were not modified. These findings suggest that LMWH may induce increased secretion of \textit{PlGF} from endothelial cells, with enhancing mobilization of endothelium-bound sFlt-1 and MPO rather than increased de novo protein production. These proteins may potentially interfere with endothelial homeostasis and NO bioavailability while bound to the endothelial cell surface by heparin sulfate.
proteoglycans. Competitive binding of LMWH may increase the circulating levels of sFlt-1 and MPO, increasing their availability for clearance and improving endothelial function. This theory would be consistent with previous conclusions that the baseline circulating levels of sFlt-1 and MPO in women with preeclampsia may only represent a portion of the total, endothelium-retained protein produced. Further studies are needed to elucidate the mechanism by which LMWH stimulates PlGF secretion from endothelial cells, as enhancing PlGF secretion may be a feasible therapeutic strategy to promote normal endothelial function.

Perspectives

The novel finding that LMWH acutely modifies the endothelial function of pregnant women at high risk of preeclampsia supports the rationale to mount an adequately powered trial to determine the effectiveness of LMWH for the prevention of early-onset preeclampsia in women at the highest-risk of this disease. Early-onset preeclampsia can be predicted with reasonable accuracy by the second trimester with multiparameter screening, providing an opportunity for the initiation of preventative therapies before the onset of clinical symptoms. In this capacity, LMWH could potentially avert serious maternal vascular dysfunction during pregnancy, leading to improved clinical outcomes. Importantly, LMWH has demonstrated a good safety profile in pregnant women with no obvious maternal or fetal side effects or major bleeding episodes. LMWH has not been proven consistently efficacious in previous trials for the prevention of all types of preeclampsia. However, this may be because of differences in the pathophysiology of early and late preeclampsia. Furthermore, the inclusion criteria of trials recruiting women at risk of severe preeclampsia have been wide and may not have resulted in consistently high-risk cohorts. The focus of future research in this context should be clinical trials that specifically define the extent of any benefit of LMWH for the prevention of severe preeclampsia, exclusively recruiting women at high risk of severe early-onset preeclampsia characterized by vascular dysfunction.

We acknowledge that our study has several limitations. Among women in our high-risk group, only 29% developed preeclampsia, although 75% did have adverse pregnancy outcomes related to placental dysfunction. This may be in part because of the fact that some women were delivered pre-emptively because of the development of coexisting intrauterine growth restriction before they meeting current diagnostic criteria for preeclampsia. It is also important to note that, although preeclampsia can be predicted with reasonable accuracy, it is a heterogeneous disease, and the relative contributions of risk factors are not known. Additionally, the effects of LMWH on maternal cardiovascular was assessed for an acute time period after one administration of the agent; further longitudinal studies are thus warranted to evaluate whether the acute treatment effects demonstrated are maintained for a longer period of time.

Conclusions

In conclusion, this present study demonstrates significant cardiovascular impairments in pregnant women at high risk of preeclampsia and, for the first time in pregnant women, provides evidence that LMWH acutely modifies endothelial function in a manner that is predicted to improve maternal vascular function. These novel in vivo data were supported by in vivo circulating protein findings and in vitro endothelial function data. Our current results are consistent with numerous previous clinical, human, animal, and cell trials, which have demonstrated beneficial vascular effects of LMWH. We think that these findings justify an adequately powered trial to assess the effectiveness of LMWH for the prevention of severe preeclampsia in screen-positive women who demonstrate vascular dysfunction.

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Disclosures

None.

References


**Novelty and Significance**

**What Is New?**

- Low molecular weight heparin acutely improves endothelial function in pregnant women who are at high risk of developing preeclampsia, a hypertensive disorder of pregnancy characterized by systemic maternal endothelial dysfunction.

**What Is Relevant?**

- Low molecular weight heparin may be an effective therapy for the prevention of preeclampsia in pregnant women who demonstrate endothelial dysfunction.
Low Molecular Weight Heparin Improves Endothelial Function in Pregnant Women at High Risk of Preeclampsia

Kelsey McLaughlin, Dora Baczyk, Audrey Potts, Michelle Hladunewich, John D. Parker and John C.P. Kingdom

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LOW-MOLECULAR WEIGHT HEPARIN IMPROVES ENDOTHELIAL FUNCTION IN PREGNANT WOMEN AT HIGH-RISK OF PREECLAMPSIA

Kelsey McLaughlin BSc1,2,3,  
Dora Baczyk MSc2,  
Audrey Potts2,  
Michelle Hladunewich MD MSc4,  
John D Parker MD1,3,  
John CP Kingdom MD2,5

1Department of Medicine, Division of Cardiology, Mount Sinai Hospital, University of Toronto, Canada  
2The Centre for Women’s and Infant’s Health at the Lunenfeld-Tanenbaum Research Institute, Mount Sinai Hospital, Canada  
3Department of Pharmacology and Toxicology, University of Toronto, Canada  
4Division of Nephrology and Obstetrical Medicine, Sunnybrook Health Sciences Centre, University of Toronto, Toronto, Canada  
5Department of Obstetrics and Gynaecology, Division of Maternal-Fetal Medicine, Mount Sinai Hospital, University of Toronto, Canada

For correspondence  
Dr. John Kingdom, MD  
Department of Obstetrics & Gynaecology  
Mount Sinai Hospital  
600 University Avenue 3-904  
Toronto, ON, Canada M5G 1X5  
Tel: 416 586-8764  
Fax: 416 586-8565  
Email: jkingdom@mtsinai.on.ca
Methods

Selection of Low- and High-Risk Pregnant Subjects

Inclusion criteria of low-risk women included: pre-viable singleton pregnancy at 22-26 weeks’ gestation, normal placental biochemistry as indicated through the integrated prenatal blood screen (IPS; normal findings include pregnancy-associated protein A [PAPP-A] >0.35 multiple of the median [MOM], alphafetoprotein [AFP] <2.0 MOM, inhibin <3.0 MOM, total chorionic gonadotropin (tbhCG <4.0 MOM), nuchal translucency <3.0 mm, first pregnancy or obstetrical history of full-term births (delivery > 36 weeks). Exclusion criteria included: vaginal bleeding prior to 12 weeks’ gestation, history of chronic illness (ie. hypertension), significant obstetrical complications in prior pregnancies (e.g preeclampsia, IUGR, abruption), >1 termination of pregnancy, abnormal placental biochemistry (indicated by one or more abnormal IPS values) or abnormal placental morphology.

Inclusion criteria of high-risk women included pre-viable singleton pregnancy at 22-26 weeks’ gestation along with two of the following four inclusion categories: 1) abnormal placental biochemistry (indicated by one or more abnormal IPS values); 2) abnormal placental shape and/or texture (maximum placental length <5th percentile, maximum placental thickness >4 cm or multiple areas of abnormal texture indicating thrombotic damage); 3) abnormal uterine artery Doppler (mean pulsatility index of left and right vessels >95th percentile); 4) abnormal clinical risk factor score (where two or more of the following criteria were met: maternal age >35, BMI 30-35, African-American ethnicity, 1st degree family history of PE, 1st degree of family history of coronary heart disease or stroke at age <60, vaginal bleeding >5 days in 1st trimester, first pregnancy or pregnancy with new partner, donor gamete/s, previous stillbirth or delivery <34 weeks with preeclampsia or intrauterine growth restriction (IUGR), maternal medical co-morbidities, diabetes requiring insulin, auto-immune disease or chronic renal disease). These inclusion criteria were based on previous work from our group and the clinical risk factors identified by the SCOPE cohort study; it was predicted that these inclusion criteria would generate an incidence of placental mediated complications of pregnancy of > 30% in the high-risk cohort1-5. Exclusion criteria included: multifetal pregnancy, major fetal abnormality, 11-13 week nuchal translucency >3.0 mm, preterm labor, preterm premature rupture of membranes, rescue cerclage, chronic hypertension on treatment <20 weeks’ gestation, current anti-hypertensive treatment, current or previous smoker, BMI >35kg/m², any perinatal concern (short cervix, low amniotic fluid, recent vaginal bleeding, intra-uterine hemorrhage), significant anemia (hemoglobin <100g/L), significant thrombocytopenia (platelets <80x10⁹/L), ongoing heparin drug therapy, hypersensitivity to enoxaparin.

Methods of Radial Artery FMD and FMC Measurement

End-diastolic, ECG-gated, longitudinal, B-mode images of the radial artery 10-15 cm below the antecubital fossa were acquired (Vivid 7, GE Healthcare, Canada; M12L) and stored for offline analysis (low-risk, n=17; high-risk, n=23). A pneumatic cuff was placed distal to the site of the probe, at wrist level. Radial artery diameter was continuously recorded throughout the baseline period (5 minutes), cuff inflation (250mmHg for 4.5 minutes) and following wrist cuff deflation (4.5 minutes). Files of imaging sequences were coded and subsequently analyzed in a blinded fashion, where the operator was not aware of either the treatment group or timepoint. FMD was calculated as the maximum percent change in radial artery diameter following wrist cuff release compared to baseline radial artery diameter. FMC was calculated as the maximum percent
change in radial artery diameter during wrist cuff inflation compared to baseline radial artery diameter. Due to equipment issues, two low-risk subjects did not have endothelial measures captured. During this analysis, three additional subjects (two high-risk subjects, one low-risk subject) were found to have poor-quality images at at least one timepoint and, because of this, their images were not included in the subsequent analysis. Our laboratory has previously reported the repeatability (range of variation) and intraclass correlation coefficient of the FMD technique as 1.7% and 0.68, respectively6. We were unable to assess endothelium-independent vasodilation with nitroglycerin due to safety concerns in pregnant women and uncertain fetal effects.

Non-Invasive Cardiac Output Monitoring (NICOM)
Non-invasive measures of cardiac output (NICOM) were utilized to assess maternal hemodynamics through bioreactance technology analysis of relative phase shifts of an alternating electrical current traversing the thoracic cavity (Cheetah Medial, Vancouver, WA; low-risk, n=19; high-risk, n=25). Four NICOM electrodes were placed on the subject’s thorax while lying semi-recumbent, two sensors below the clavicle and two sensors at the costal margin. Stroke volume is derived through analysis of relative phase shifts of an alternating 75 kHz electrical current between sensors. These phase shifts have been reported to be correlated to stroke volume7, 8. Sensor electrocardiogram reading allows for detection of heart rate. Hemodynamic monitoring was continued for 15 minutes. Equipment issues prevented NICOM to be captured in one low-risk subject.

Blood & Urine Collection
Venous blood samples were collected by venipuncture in the low-risk group and through a 5F cannula in the high-risk group, both in the antecubital vein (low-risk, n=18; high-risk, n=24). Plasma was collected into tubes containing EDTA and sodium citrate, while serum was collected into buffer-free tubes and serum-separating tubes. Samples were immediately sent for complete blood count, cholesterol, creatinine, uric acid and liver function tests as part of routine clinical assessment. Plasma samples were then spun at 4000 rpm for 10 minutes; a subset of plasma samples underwent an additional spin at 1300 rcf for 2 minute for platelet-poor plasma. Serum samples were spun at 2000 rcf for 10 minutes. The supernatant was aliquoted and stored at –80°C. Urine samples were collected whole and stored at –80°C.

Plasma Clotting Time
The activated partial thromboplastin time (APTT) assay was utilized for quantification, and detection of deficiencies, of all plasma intrinsic coagulation factors except for Factor VII (Pacific Hemostasis, Thermo Scientific, Hudson, NH). Plasma was mixed with APTT-XL reagent (low-risk, n=16; high-risk, n=20), which contained a plasma activator and phospholipid, and this mixture was then mixed for 3 minutes at 37°C. Calcium chloride was then added and the time to clot formation was determined by measuring sample turbidity at 405nm.

In Vitro Endothelial Cell Functional Assays
HUVECs were utilized for the analysis of in vitro endothelial effects of patient serum and LMWH. This cell line maintains numerous features of in vivo endothelial cells, including the expression of cell specific markers, such as CD31 and von Willebrand factor, and is able to
appropriately respond to stimuli, such as VEGF\textsuperscript{9\textendash}11. Although not a maternal cell line, HUVECs have been widely used for experiments examining the endothelial cell function through the effects of various stimuli on endothelial cell angiogenesis, proliferation, toxicity, gene expression and protein production\textsuperscript{11,12}.

**In Vitro Angiogenesis**

The effects of subject serum and LMWH treatments on HUVEC angiogenesis were assessed. Growth-factor reduced Matrigel (BD Biosciences, Oxford, UK) was thawed at 4°C, 50uL of Matrigel was added to wells of a 96-well plate and incubated at atmospheric 95% O\textsuperscript{2} /5% CO\textsubscript{2} at 37°C for an hour to solidify. Next, 100uL of EBM based media (Lonza) with EGM-2MV bullet kit (Lonza) was added into each well containing 20,000 HUVECs/well. Serum treatment were added to each well (low-risk, n=6; high-risk, n=5), as follows: 1) 5% FBS, 2) serum-free media (SFM) with 5% low-risk subject serum, 3) SFM with 5% low-risk subject serum and LMWH (2.5 units/mL), 4) SFM with 5% high-risk subject serum, 5) SFM with 5% high-risk subject serum and LMWH (2.5 units/mL). The LMWH concentration of 2.5 units/mL was chosen for this assay, as it is equivalent to maternal circulating plasma levels of LMWH within the therapeutic range of 10,000 units/day\textsuperscript{13,14}. The plates were incubated for 24 hours at atmospheric 95% O\textsuperscript{2} /5% CO\textsubscript{2} at 37°C. The wells were photographed under 4x magnification with a Qimaging Micropublisher 5.0 RTV camera coupled with a Leica DMIL LED microscope and coded. Analysis was performed on coded images, blinded to the background experiment. The capillary-like structures formed by differentiating HUVECs were quantified through measuring the total tubule length and number of branch points in each well. Data was presented as the average of duplicate samples and normalized to FBS control. As results were similar between parameters, only tubule length data is shown.

**In Vitro Proliferation**

The effects of subject serum and LMWH treatments on HUVEC proliferation were assessed using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) according to the manufacturers’ instructions. HUVECs were plated in 96-well plates and cultured for 24 hours in media at atmospheric 95% O\textsuperscript{2} /5% CO\textsubscript{2} at 37°C. Cells were then treated with one of the following treatments and cultured for 24 hours: 1) 5% FBS, 2) SFM with 5% low-risk subject serum (n=3), 3) SFM with 5% high-risk subject serum (n=3), 4) 5% FBS with LMWH (2.5 units/mL). 20uL of the CellTiter 96\textsuperscript{©} Aqueous One Solution Reagent into each well and incubated at atmospheric 95% O\textsuperscript{2} /5% CO\textsubscript{2} at 37°C for 3 hours. Absorbance was then read at 490nm. Data was presented as the average of triplicate samples and normalized to FBS control.

**In Vitro Toxicity**

The effects of subject serum and LMWH treatments on HUVEC toxicity were assessed using the CytoScan™ SRB Cytotoxicity Assay (G Biosciences, Maryland Heights, MO). HUVECs were plated in 96-well plates and cultured for 24 hours at atmospheric 95% O\textsuperscript{2} /5% CO\textsubscript{2} at 37°C. Cells were then treated with one of the following treatments and cultured for 24 hours: 1) 5% FBS, 2) SFM with 5% low-risk subject serum (n=3), 3) SFM with 5% high-risk subject serum (n=3), 4) 5% FBS with LMWH (2.5 units/mL). Cells were washed with trichloroacetic acid and water then left to dry overnight. 50uL of 0.4% Sulforhodamine B solution was added to the wells for 30 minutes to stain the cells and washed with 1% acetic acid, then left to dry overnight.
Finally, Sulforhodamine B Assay Solubilization Solution was added to wells and absorbance was read at 565nm. Data was presented as the average of triplicate samples and normalized to FBS control.

# Quantitative RealTime PCR Procedures and Primer Sequences

HUVEC cells were plated in 24-well plates and cultured for 24 hours in media containing subject serum (low-risk, n=6; high-risk, n=4), as follows: 1) 5% FBS, 2) SFM with 5% low-risk subject serum, 3) SFM with 5% low-risk subject serum and LMWH (2·5 units/mL), 4) SFM with 5% high-risk subject serum, 5) SFM with 5% high-risk subject serum and LMWH (2.5 units/mL). The RNeasy Plus Mini Kit (Qiagen) was used to extract total RNA and 500 ng of mRNA was reverse transcribed using iScript Reverse Transcription Supermix (Bio-Rad, Mississauga, ON, Canada), according to manufacturers’ instructions. The following protocol was utilized for reverse transcription: 25°C for 5 minutes, 42°C for 30 minutes, 85°C for 5 minutes. Quantitative real-time PCR (qRT-PCR) was then performed to evaluate HUVEC gene expression using LuminoCt SYBR Green qPCR ReadyMix (Sigma-Aldrich) on the CFX384 Real-Time PCR Detection System (Bio-Rad). The following primers were used: PlGF-1 forward primer: 5’-CCAGCAGTGGGGCCTTGTCT-3’; PlGF-2 forward primer: 5’-CCAGTGGGCCCTGTCTGCTG-3’; PlGF1/2 reverse primer: 5’-ACACTTCTGGAGGGGTACCA-3’. The following protocol was utilized for the qRT-PCR reactions: activation at 95°C for 2 minutes, denaturation over 40 thermal cycles at 95°C for 5 seconds, annealing/extension at 60°C for 30 seconds. Gene expression was normalized to the geometric mean of the housekeeping genes TBP (forward primer: 5’-TGCACAGGAGCCAAGAGTGAA-3’; reverse primer: 5’-CACATCACAGCTCCCCACCA-3’), YWAHZ (forward primer: 5’-ACTTTTGGTGATCATTGCTTCCA-3’; reverse primer: 5’-CGCCCAGCAGAAACCAGTAT-3’) and TOP1 (forward primer: 5’-GATGAAACCTGAGATGAGGC-3’; reverse primer: 5’-TCAGCATCATCCTCATCTCG-3’). All data were expressed as a fold change relative to the FBS control.

# Intracellular and Extracellular Levels of PlGF

HUVEC cells were plated in 24-well plates and cultured for 24 hours with serum samples (low-risk, n=3; high-risk, n=3), as follows: 1) 5% FBS, 2) SFM with 5% low-risk subject serum, 3) SFM with 5% low-risk subject serum and LMWH (2.5 units/mL), 4) SFM with 5% high-risk subject serum, 5) SFM with 5% high-risk subject serum and LMWH (2.5 units/mL). Media was collected and spun at 2000 rcf for 10 minutes; supernatant was then stored at –80°C. Cells were washed with PBS and lysed with 250uL of a provided cell lysis buffer (R&D Systems, Minneapolis, MN) for 1 hour. Following a spin of 1000 rcf for 15 minutes, the cell lysis was stored at –80°C. The cell lysates were quantified for PlGF and media samples were quantified utilizing ELISA for PlGF, sFlt-1 and VEGF (R&D Systems, Minneapolis, MN) according to manufacturers’ instructions. Results were normalized to the levels of protein in the respective original serum treatment and presented as relative to FBS.
References


Table S1. Inclusion criteria for pregnant women at 24 weeks’ gestation considered high-risk of developing placenta-mediated complications. 7 (28%), 14 (56%) and 4 (16%) met 2, 3 or all inclusion criteria categories, respectively. Data are presented as n (%).

IPS indicates integrated prenatal screening.

<table>
<thead>
<tr>
<th>Inclusion Criteria</th>
<th>High-Risk Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abnormal IPS</td>
<td>15 (60)</td>
</tr>
<tr>
<td>Abnormal placental morphology</td>
<td>21 (84)</td>
</tr>
<tr>
<td>Abnormal uterine artery Doppler</td>
<td>20 (80)</td>
</tr>
<tr>
<td>Positive clinical risk factor score</td>
<td>16 (64)</td>
</tr>
</tbody>
</table>
Table S2. Clinical risk factor inclusion criteria for pregnant women at 24 weeks’ gestation considered high-risk of developing placenta-mediated complications. Data are presented as n (%).

<table>
<thead>
<tr>
<th>Clinical Risk Factor</th>
<th>High-Risk Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age &gt;35</td>
<td>13 (52)</td>
</tr>
<tr>
<td>BMI 30-35</td>
<td>7 (28)</td>
</tr>
<tr>
<td>African-American descent</td>
<td>3 (12)</td>
</tr>
<tr>
<td>1st degree family history of preeclampsia</td>
<td>2 (8)</td>
</tr>
<tr>
<td>1st degree family history of coronary heart disease/stroke at age &lt;60</td>
<td>3 (12)</td>
</tr>
<tr>
<td>Vaginal bleeding &gt;5days in 1st trimester</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Pregnancy with new partner or primigravida (no prior pregnancy)</td>
<td>7 (28)</td>
</tr>
<tr>
<td>Donor gamete/s (egg and/or sperm)</td>
<td>1 (4)</td>
</tr>
<tr>
<td>Previous placental abnormalities, unexplained stillbirth, or delivery &lt;34 weeks with IUGR or PE</td>
<td>12 (48)</td>
</tr>
<tr>
<td>Diabetes (any type) requiring insulin</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Auto-immune disease</td>
<td>1 (4)</td>
</tr>
<tr>
<td>Chronic renal disease</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

BMI indicates body mass index; IUGR, intrauterine growth restriction; PE, preeclampsia.
Table S3. Radial artery diameters, blood flow changes and reactive hyperemia data. Data are presented as mean ± SEM.
Figure S1. Plasma and urine angiogenic factors of pregnant women at low- and high-risk of PE at 24 weeks’ gestation. High-risk pregnant (n=24) women demonstrated significantly lower levels of plasma and urine PI GF (A, B) and higher plasma levels of sENG (C) when compared to low-risk pregnant women (n=18). There were no significant differences in plasma levels of sFlt-1 (D), MPO (E) or ET-1 (F) between the two groups. Data are presented as medians ± 25th and 75th percentile confidence intervals. *p < 0.05 and **p=0.0001.
Figure S2. Correlation between plasma PI GF and total peripheral resistance (A; \( r=-0.61, P<0.0001 \)) and uterine artery PI (B; \( r=-0.58, P=0.0005 \)) at 24 weeks’ gestation in the combined set of data from pregnant women at low-risk (open circle) and high-risk (closed circle) of PE at 24 weeks’ gestation.
Figure S3. Plasma clotting time of pregnant women at high-risk of developing placental dysfunction randomized to placebo (n=9) or LMWH (n=10). Women randomized to LMWH demonstrated significantly longer clotting times following 3-hours post-infusion compared to baseline. Data are mean ± SEM. *p < 0.05. LMWH=low molecular weight heparin.