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DYSREGULATION OF PROMYELOCYTIC LEUKEMIA (PML) PROTEIN EXPRESSION IN PREECLAMPTIC PLACENTAE

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Abstract

Promyelocytic leukemia (PML) protein is a nucleoprotein that can regulate a variety of cellular stress responses. The aim of this study was to determine qualitative and quantitative changes in PML expression in preeclamptic placentae. Immunoblot, qRT-PCR and immunohistochemistry techniques were used to determine PML gene expression and localization in normal (n=6) and preeclamptic (n=6) placentae and primary cells. PML protein was immunolocalized within nuclei of villus mesenchyme, but largely absent in trophoblast nuclei, with a trend for increased PML reactivity in preeclamptic placenta. Immunoblot analyses of nuclear extracts confirmed relative increases (~3 fold) of PML expression in preeclamptic placentae (p<0.05). Conversely, less PML mRNA (~2 fold) was detected in preeclamptic versus normal placental samples. In vitro, PML expression could be increased by hypoxia in cultured endothelial cells but not trophoblast. Increased PML protein expression in preeclamptic villi suggests it could contribute to decreased vascularity and placental growth and/or function.

Keywords

endothelial cells; preeclampsia; promyelocytic leukemia protein; PML; trophoblast

INTRODUCTION

The clinical syndrome preeclampsia affects 5–10% of primiparous women, and remains one of the leading causes of maternal and perinatal morbidity and mortality¹. Although the etiology of preeclampsia remains unclear, its pathophysiology is thought to derive from abnormal placentation resulting in a poorly perfused placental bed (see²) and the development of various cellular stresses against the developing fetus³. Several systemic and/or localized vascular defects have been noted in preeclampsia including generalized endothelial dysfunction^{4,5}, increased senescence of endothelial progenitor cells⁶, and an overall anti-angiogenic state^{7–9}. Elucidating stress-response proteins that could affect angiogenic responses may therefore be helpful in understanding the pathophysiology of preeclampsia.

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Promyelocytic leukemia (PML) protein was originally identified in patients with acute promyelocytic leukemia¹⁰. A number of PML transcripts are produced via alternative splicing¹¹, producing seven protein isoforms (PML I-VII)¹². Each PML isoform contains an N-terminal Ring finger, two B-boxes, and coiled-coil domain that designate PML as a member of the TRIM family of proteins¹³, and which enables multiple post-translational modifications¹⁴. PML exists primarily as a nucleoprotein, expressed in most cells, and exhibits multifunctional characteristics^{15;16}. PML can regulate transcription, translation, apoptosis, cell cycle progression, DNA repair, anti-viral replication, cell senescence¹⁷, and has anti-angiogenic¹⁸ and tumor suppressive potentials¹⁹. The ability of PML to regulate such diverse cellular functions is likely due to its sequestration of target proteins into PML nuclear bodies²⁰ coupled with its expression as several different post-translationally modified isoforms, each with potentially different functions^{17;21}.

Expression of PML within villi of normal placentae was described by Kim et al²² to be most notable in fibroblasts, endothelial cells of fetal stem vessels and macrophages. Temporally, expression of nuclear PML tends to increase with gestational age and remains high from approximately 31 weeks gestation to term and suggests that PML contributes to regulating growth and development of the placenta²². Given the associations between cellular stresses presumed to occur in the preeclamptic placenta and the importance of nuclear PML in regulating a number of diverse and vital cell stress responses, including vascular development/function, we sought to characterize PML localization patterns and relative expression changes between normal and preeclamptic placentae.

MATERIALS AND METHODS

Patient selection

Control placentae (n = 6) were collected from women who remained normotensive throughout pregnancy and whose infants were not considered intrauterine growth restricted. Preeclamptic placentae (n = 6) were collected from women meeting established criteria for preeclampsia¹. All pregnancies were singletons, and no other fetal or maternal complications including renal disease, cardiovascular disease, or chronic maternal hypertension were evident. To avoid potential stress effects of labor and delivery²³ on PML expression, all placentae were collected from women without labor and immediately after Cesarean section. Indications for Cesarean section in the normal control group were previous Cesarean section or malpresentation. Placentae collection protocols were approved by the Springfield Committee for Research Involving Human Subjects at Southern Illinois University School of Medicine.

Following Cesarean section, placenta samples were immediately obtained from three separate locations on the maternal aspect of the placenta to determine regional differences in PML expression. One sample was collected from a central cotyledon (cotyledon 1), a peripheral cotyledon (cotyledon 3) and a paracentral cotyledon situated between these two sites (cotyledon 2). Obvious areas of vascular or villus pathology were avoided. Maternal decidua was removed from samples, the remaining villus tissue was briefly rinsed in sterile saline and fixed in 4% paraformaldehyde for immunohistochemistry. Adjacent samples were similarly collected for protein and RNA extraction, frozen on dry ice and stored in liquid nitrogen for protein studies, or placed in RNeasy lysis buffer (Qiagen, Crawfordsville, IN) and stored at -20°C for RNA studies.

Immunohistochemistry

Placental biopsies were paraffin embedded and 5µm sections were deparaffinized in d-citruelline, rehydrated in a graded series of water alcohol, then washed in phosphate buffered

saline (PBS, pH 7.2). Antigen retrieval was accomplished by boiling sections in 10mM citrate buffer (pH 6.0) for 5 minutes and allowed to cool to room temperature for 20 minutes. Sections were blocked for 1hr with 3% BSA/PBS and incubated with pre-immune rabbit IgG or rabbit polyclonal antibody against a conserved region common to all human PML protein isoforms (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:250 in 0.3% BSA/PBS overnight at 4°C. PML immunoreactivity was detected with Alexa-fluor 568-conjugated goat anti-rabbit IgG antibody (1:400, Molecular Probes, Carlsbad, CA) for 2 hours at room temperature. Slides were washed in PBS and coverslipped with Vectashield Hardset mounting media (Vector Laboratories, Burlington, CA). Immunofluorescence was captured using an Olympus Scope-pro fluorescent microscope and image-pro 5.0 software. Threshold exposure times and camera settings were held constant to those obtained with non-immune rabbit IgG immunofluorescence.

Immunoblots for PML expression

Villus tissue samples were thawed in PBS supplemented with complete protease inhibitor cocktail (Calbiochem, San Diego, CA) and phosphatase inhibitors. Tissue homogenization and nuclear fractionation were performed according to instructions (Nuclear Extract Kit, Active Motif, Carlsbad, CA) as previously described²⁴ and protein concentrations in the lysates were determined using D_C Protein Assay (Bio-Rad, Hercules, CA). Nuclear lysates (30ug) were resolved on 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% BSA Tris-buffered saline containing 0.05% Tween-20 (TBST), and incubated with rabbit anti-PML antibody (Santa Cruz Biotechnology) (1:1000) diluted in 1% BSA-TBST overnight at 4°C. Membranes were washed and incubated with anti-rabbit HRP-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) (1:5000). PML immunoreactivity was detected by chemiluminescence (Amersham Biosciences, Piscataway, NJ). We found that nuclear lamin A/C protein expression can fluctuate under changing O₂ conditions (data not shown). However, β -actin is expressed in the nuclear compartment²⁵ and does not fluctuate under hypoxia which enables us to normalize relative PML densitometry values to β -actin reactivity as described²⁴. Quantification of PML reactivity was determined by comparing ratios of PML/ β -actin for each cotyledon in PE to ratios from a matched normal placenta analyzed on the same membrane.

Quantitative RT-PCR

One microgram of total RNA was reverse transcribed into cDNA using iScript (Bio-Rad) according to the manufacturer's instructions. Respective sense and antisense primers for the human genes analyzed are: GAPDH: 5'-GAAGGTGAAGGTCGGAGTCAA-3' and 5'-CAGAGTTAAAAGCAGCCCTGG-3'; GCM-1: 5'-GTCAGGCTCTCAAATC-3' and 5'-CCACTCTAGTATTTCCACCC-3'; PGF: 5'-AGAAGATGCCGGTCATGAG-3', and 5'-ACACTTCCTGGAAGGGTAC-3'; PML: 5'-CTACGCTGACCAGCATCTAC-3', and 5'-CTGATGTGCGCACTTGAGCTC-3'; sflt-1: 5'-AGGGGAAGAAATCCTCCAGA-3', and 5'-CAACAAACACAGAGAAGG-3'. Specificity of the primers and validity of expected amplicons were confirmed by gel electrophoresis and primers for PGF, PML and sflt-1 were designed to recognize conserved regions of each target (data not shown). QRT-PCR was performed using iQSYBR Green PCR supermix (Bio-Rad) and included a melt curve analyses after each qRT-PCR reaction to confirm product integrity. Relative changes in gene expression between the preeclamptic and normal samples were determined using the $\Delta\Delta CT$ method²⁶ as described²⁴.

In vitro manipulation of PML expression in HUVEC and primary trophoblast

Human cytotrophoblast and umbilical vein endothelial cells (HUVEC) were isolated from normal term placentae as previously described^{24,27}. Cell cultures were treated under

hypoxic conditions (~1% O₂) as previously described²⁴ or treated with rhInterferon- β (200 units/ml) for 24 hours. Interferon- β is a well established inducer of PML expression²⁸.

Nuclear protein extracts were processed for immunoblotting as above. For immunohistochemical studies, parallel cultures were fixed with ice cold acetone/methanol (50% v/v) and blocked with 2% BSA/PBS. Rabbit anti-PML or non-immune IgG (1:250) in 2% BSA/PBS was reacted overnight at 4°C. Cells were rinsed and PML immunoreactivity detected with Alexafluor 568 conjugated secondary antibody and photographed as above.

Statistical analyses

Nonparametric Mann-Whitney tests were used to compare clinical parameters between the normal and preeclamptic women at delivery. Wilcoxon signed rank test was used for the relative differences in PML expression by western blot between the normal and preeclamptic samples and Kruskal-Wallis nonparametric ANOVA was used to evaluate expression differences between cotyledon samples. A one sample T-test was used to analyze relative differences in gene expression between the normal (set to 100) and the preeclamptic samples. A p value ≤ 0.05 was considered statistically significant.

RESULTS

Clinical characteristics of normal and preeclamptic pregnancies are summarized in Table 1. There was no significant difference in maternal age or maternal body mass index between the two study groups. Maternal systolic and diastolic blood pressures were significantly higher in the preeclamptic group at delivery. Mean gestational age in the normotensive patients was ~39 weeks while the preeclamptic patients were delivered at a significantly earlier gestational age (~31 weeks). Because of the earlier gestational age at delivery, there was a significant difference in birth weights between the groups.

Nuclear PML was localized primarily within the fetal stem vessel endothelial cells and stroma cells of the villi (Figure 1) with little to no PML immunoreactivity in villus cytotrophoblast or syncytiotrophoblast. These findings are in qualitative agreement with previous studies²². In placentae from normal term deliveries, the number and distribution of cells showing nuclear PML body expression was fairly uniform between different regions of the same placenta. In contrast, preeclamptic placentae exhibited varied regional differences in PML. There also appeared to be relatively greater PML immunoreactivity in preeclamptic placenta than in normal samples (Figure 1), specifically in vascular endothelial and stroma cells. The relative differences in PML expression are more evident upon comparison between the central, paracentral and peripheral regions of the placenta. There were relatively smaller differences observed between the paracentral (Figure 1B) cotyledons of preeclamptic samples and comparative sections from normal placentae. However, there was a trend for increased visual differences of PML expression within sections collected from the central and peripheral cotyledons of the preeclamptics (Figure 1A and C).

PML nuclear bodies from preeclamptic and normal placentae localized as diffuse or speckled patterns within nuclei. Particularly notable was the heterogeneous PML nuclear body distribution patterns within endothelial cells of the fetal stem vessels. Some trophoblast nuclei sequestered within syncytial knots demonstrated very weak PML nuclear body immunoreactivity (data not shown), however, this reactivity did not seem to significantly vary between normal and preeclamptic placentae.

In that density differences between samples could influence interpretation of the immunohistochemistry findings, we employed a more quantitative immunoblot approach to confirm differences between the clinical samples (Figure 2A). Multiple bands corresponding to potentially different PML isoforms or modified isoforms were detected. These findings

are typical for PML protein expression as alternative splicing of the PML gene results in several specific PML protein isoforms and post-translational modifications of PML produce multiple banding patterns²¹. PML bands between ~60–120 kDa were used for quantitative analyses between samples. Normalized PML/ β -actin ratios in PE were compared to normalized ratios from control placentae analyzed on the same blot. PML expression was elevated in central, paracentral and peripheral regions of PE placentae with each single region demonstrating some variation (Figure 2B). However, variations between regions were not significantly different (Kruskal-Wallis ANOVA, $p > 0.5$). Mean normalized PML expression across all regions in PE placentae was ~3 fold higher ($p = 0.03$) than in control placentae (Figure 2B). Thus, the western blot data generally supports the more subjective spatial differences detected by immunohistochemistry.

Real time RT-PCR was used to assess differences in PML mRNA expression between the normal and preeclamptic placentae (Figure 3). As control, differences in mRNA expression of genes known to be dysregulated in preeclampsia were also determined. Despite increased PML protein detected by immunoblots, PML mRNA expression was significantly decreased in preeclamptic samples. Relative mRNA expression of the angiogenic growth factor, placenta growth factor (PGF), and its transcriptional regulator in trophoblast, glial cells missing-1 (GCM1)²⁴ were also significantly decreased in the preeclamptic samples as described^{29;30}. In contrast, relative mRNA expression of the soluble receptor for PGF and VEGF (sflt-1 or sVEGFR1) was significantly increased as expected³¹. The control genes results in preeclamptic samples confirm the validity of the PML mRNA findings.

Given the presumed association between hypoxic stress and preeclampsia, we determined if low oxygen tension could contribute to increased PML protein expression in vascular endothelial cells or if PML expression could be induced in primary trophoblast (Figure 4). PML bodies were readily evident in HUVEC cultured under standard conditions (Figure 4A). Exposure to interferon- β , a known inducer of PML²⁸, produced a marked increase in the expression and nuclear organization of the PML bodies. Low O₂ tension appeared to increase total PML immunoreactivity, however PML nuclear bodies were less structured than those induced by interferon- β . In contrast, primary trophoblast exhibited very little nuclear PML expression, which is in agreement with the placental immunohistochemistry findings (Figure 1). Exposure of trophoblast to interferon- β or low oxygen conditions did not significantly alter PML body immunoreactivity (Figure 4A).

Immunoblotting was performed on nuclear extracts from these cultures to more accurately assess quantitative changes in PML expression (Figure 4B). Exposure of HUVEC to 1% O₂ resulted in an increase in total PML expression with strong induction of isoforms at ~60Kd and ~115Kd, qualitatively similar to that induced by interferon- β . Primary term trophoblast exhibited less nuclear PML protein expression than HUVEC (the trophoblast blot is overexposed to highlight comparisons between treatments within these cells). Importantly, in contrast to the responses in HUVEC, little alteration in expression and/or distribution of PML in primary trophoblast could be detected. There was a relatively small increase in the major PML band at ~75Kda, however, neither interferon- β nor hypoxia resulted in induction of the ~115Kda or ~60Kda PML isoforms that were induced by these treatments in the HUVEC cultures. The immunoblotting findings are in agreement with the immunohistochemical findings indicating that PML expression patterns within the trophoblast remain relatively consistent. Collectively, these results suggest that stressors such as low oxygen tension and type I interferons can increase PML expression in vascular endothelial cells. In contrast, trophoblast express little endogenous PML and they are relatively impervious to PML induction by these stressors.

DISCUSSION

Preeclampsia is often reported to be associated with exposure of the placenta to various stress conditions, in particular hypoxic stress³². The goal of this investigation was to compare the expression and distribution patterns of a significant stress response protein, PML, in preeclamptic placentae. Increased PML protein expression was identified via immunohistochemical analyses in the placentae of preeclamptic patients relative to normotensive term control placentae. Increased PML immunoreactivity was most noticeable within cells of the villus core (macrophages, endothelial cells, fibroblasts) rather than in trophoblast cells. Indeed, little PML reactivity could be detected within trophoblast cells in vivo or in vitro. Increased PML isoform expression was evident in nuclear extracts of preeclamptic placentae in spite of less PML mRNA being detected. In vitro, PML expression and nuclear organization can be altered in endothelial cells, but not trophoblast, by low oxygen or interferon- β .

Our study is consistent with previous reports²² in that PML expression is detectable in normal placenta localizing within diffuse or speckled nuclear bodies throughout gestation. This heterogeneous morphology is limited to specific cell-types of the villus stroma, including capillary endothelial cells, villus stromal fibroblasts, and resident macrophages but not villus cytotrophoblast or syncytiotrophoblast²². Outside of the villi, PML protein expression had been described in amnion cells, trophoblast giant cells and intermediate trophoblast within the deciduas²². We did not have an opportunity to study these extravillous locations in our archived samples.

Temporally, PML protein expression in normal placentae has been reported to increase as gestation proceeds to term²². Our study extends these observations by including preeclamptic samples. Whereas PML protein levels were very low at less than 31 weeks gestational age in normal placentae²², our PE samples at 28–34 weeks, demonstrated relatively greater nuclear PML protein expression than did control samples. Therefore, we anticipate that differences in PML expression could be larger than depicted here if normal placenta samples from 28–34 weeks gestation were available for study. Because stresses of labor may induce changes in PML expression, our study examined PML expression in preeclamptic placentae from unlabored patients. Thus, it was imperative to obtain control placentae from patients who underwent Cesarean delivery in the absence of labor. Ideally, normal placentae from scheduled Cesarean deliveries to match the gestational age as the preeclamptic patients would be preferable. However, there are very few, if any, circumstances in which normal pregnant unlabored patients are delivered by elective Cesarean delivery at the same average gestational age as the preeclamptic group. Larger studies are needed to determine if changes in PML expression correlate with severity of preeclampsia and/or whether other clinical parameters (IUGR, diabetes, smoking, etc) are associated with altered PML expression patterns in the villi.

We found a dissociation in the relative expression levels of PML protein and mRNA in the preeclamptic placenta samples. This is likely due to post-translational modifications which alter PML protein stability (see³³). For example, PML protein can be phosphorylated and/or sumoylated and protected from degradation and exogenous stimuli have been shown to increase or decrease PML protein levels independent of mRNA levels in different cell types^{34;35}. Interestingly, we did not detect prominent levels of PML protein in isolated primary trophoblast nor could we induce PML protein expression in trophoblast with interferon- β . Post-translational modifications may explain these results. SUMO-1 extends the half-life of PML by inhibiting ubiquitination²¹. Sumoylation is also required for nuclear body formation, as PML that is diffusely localized within the nucleoplasm lack SUMO-1 modification³⁶. It has recently been shown that SUMO-1 is degraded by a highly expressed

trophoblast-specific SUMO protease, SENP-2, a protein necessary for trophoblast differentiation and placentation³⁷. Thus, we speculate that the relatively low and stable expression of PML in trophoblast may be due to SENP-2 activity. Whether post-translational modifications are responsible for altering PML protein expression and/or functional activity in the preeclamptic tissues is not known but suggest future investigations of sumoylation in preeclampsia should be considered.

Recently, it was shown that preeclamptic placentae not complicated by IUGR, are affected by increased endothelial progenitor cell senescence and reduced function⁶. Over expression of PML has been shown to cause increased fibroblast senescence³⁸ and can function to inhibit angiogenesis¹⁸. Thus, it could be that increased endothelial cell nuclear expression of PML during preeclampsia contributes to the relative senescence of these cells. Similarly, it is possible that increased expression of PML in placental macrophages (Hofbauer cells) could affect placental development and vascularity as these cells have been shown to co-localize near fetal stem vessels and express numerous growth factors, including angiogenic growth factors, that can regulate trophoblast and villus branching morphogenesis^{39;40}. Expression of the angiogenesis related genes used in this study (PGF, sflt-1) were significantly different between normal and preeclamptic samples, as expected. Although these genes were used primarily to validate the PML mRNA findings, both can be expressed in vascular endothelial cells. The possible relationship between the expression levels of these genes and changes in PML expression in preeclampsia is unknown. Nonetheless, it is conceivable that differences in PML expression or function within several different cells of the villus core may contribute to the inadequate placentation of preeclampsia. Furthermore, such effects may be independent of trophoblast in that alterations in PML expression/localization were not readily evident in these cells.

PML function is highly studied in cancer and viral infection, but its significance in other disease conditions is relatively unknown. In the context of preeclampsia, PML nuclear bodies may interfere with angiogenic responses through inhibition of mTOR activation, a mechanism which limits angiogenic responses in both neoplastic and ischemic conditions⁴¹. Additionally, it may reduce hypoxia-induced angiogenesis through inhibition of hypoxia-inducible factor 1-alpha (HIF-1 α) protein synthesis¹⁸. In correlative support of this, increased expression of HIF-1 α protein is most notable in syncytiotrophoblast of preeclamptic placentae⁴², where there is relatively little PML expression (current study and²²). Thus, ischemic stress may enable increased HIF-1 α production but PML-mediated inhibition of HIF-1 α protein translation may result in heterogenous expression of HIF-1 α protein between different cell types. Since HIF-1 α protein expression is not thought to be increased in IUGR⁴³, PML regulatory pathways should also be investigated in other obstetrical complications associated with hypoxia.

In summary, our findings agree with previous studies showing PML expression within cells of the villus core, but not trophoblast, in normal placentae²². We extended these studies to show that nuclear PML expression is elevated within these cells, but not trophoblast, in preeclamptic placentae. Furthermore, PML expression is inducible by hypoxia or interferon- β treatments in vascular endothelial cells but not trophoblast. Our results, coupled with others' showing PML protein can adversely effect hypoxia-mediated angiogenic responses¹⁸, provide a novel suggestion that increased PML expression may contribute to inadequate placental vascularity associated with preeclampsia.

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Figure 1.

Increased PML immunoreactivity in preeclampsia.

Sections of cotyledon from normal and preeclamptic placentae were reacted with anti-PML antibodies and localized with Alexa-fluor goat anti-rabbit Fab'2 secondary. Representative sections of PML immunoreactivity within the central cotyledon (panel A), a paracentral cotyledon (panel B) and a peripheral cotyledon (panel C) are shown (600x). All images were captured with camera settings identical to the non-immune IgG threshold exposure level (IgG).

Figure 2a

Figure 2b

Figure 2.

Nuclear PML protein expression in normal and preeclamptic placentae.

A. Representative immunoblot of PML expression in normal and preeclamptic placentae. Nuclear lysates were separated by SDS-PAGE, transferred to nitrocellulose and probed with anti-PML. Blots were stripped and reprobed with antibodies to β -actin to control for loading consistency. B. Increased PML expression in preeclamptic placental lysates. Normalized PML/ β -actin expression in uncomplicated pregnancies was set = 1.0 (n = 6 for each group except cotyledon 3 where n = 5). Normalized PML expression from all 3 sampled regions of the preeclamptic placenta within each region (left panel) and pooled normative values of overall PML protein expression in preeclamptic versus normal samples (right panel) are shown.

Figure 3.

Expression of PML mRNA is decreased in preeclamptic placentae. Total RNA was extracted from villus samples of normal and preeclamptic placentae and relative gene expression evaluated by real time RT-PCR for PML, PGF, GCM-1, and sflt-1. Relative gene expression in the preeclamptic placentae was normalized to levels in the pool of normal placentae. All findings were significantly different from normals (set to 100%).

Figure 4a

Figure 4b

Figure 4. Induction of nuclear PML expression in primary endothelial cells but not trophoblast in vitro. A. Immunofluorescence reactivity of PML in cells cultured in either standard conditions (21% O₂), 200U interferon-β, or 1% O₂. Cells were fixed, permeablized and reacted with antibodies to PML (100x magnification). B. Nuclear protein extracts from parallel cultures were subjected to immunoblotting with antibodies to PML as in Figure 2. Results are representative of three (HUVEC) or two (trophoblast) independent experiments with identical results.

TABLE 1

Clinical Parameters of Patients*

	Normals (6)	Preeclamptic (6)	p^a
Maternal age (yrs)	23.2 ± 3.1	21.7 ± 4.2	0.470
Gestational age (wks)	38.8 ± 0.7	31.2 ± 2.2	0.002
Maternal BMI ^b	38.0 ± 7.4	37.7 ± 8.2	0.940
Gravid	2.8 ± 1.2	1.2 ± 0.4	0.010
Para	1.2 ± 0.4	0.2 ± 0.4	0.016
Birth weight (g)	3675 ± 494	1421 ± 556	0.002
Systolic ^b (mmHg)	112.8 ± 9.8	164 ± 19.9	0.002
Diastolic ^b (mmHg)	63.2 ± 8.1	103.0 ± 13.0	0.002
Proteinuria ^c	nd ^d	6/6; 2.8 ± 0.8	

* mean ± sd; except where noted

^a Mann-Whitney test^b at delivery^c proteinuria; dip > 2.0^d not determined