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저체중아 임신에서 임신 제1분기 혈장내 Asymmetric Dimethylarginine과 태반내 항산화 효소의 발현에 관한 연구

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Elevated Plasma Asymmetric Dimethylarginine in the First Trimester and Unaltered Placental Antioxidant Enzyme Expression in Pregnancies with Small-for-gestational-age Infants

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Objectives: This study was undertaken to evaluate whether maternal plasma concentrations of asymmetric dimethylarginine (ADMA) and Glutathione Peroxidase (GPX) activity in the first trimester of pregnancy and at the time of delivery in women with small for gestational age (SGA) infants are increased and to demonstrate whether placental expression of endothelial nitric oxide synthase (eNOS) and antioxidant enzymes are changed in women with SGA.

Study design: Maternal plasma samples were collected between 10-13 weeks' gestation during prenatal visits and again at the time of admission for delivery from 20 women with uncomplicated pregnancy and 20 women with SGA. ADMA concentrations were measured using ELISA and GPX activities were measured using spectrophotometry. Placental expression of total eNOS, eNOS phosphorylation at serine^{1177}, MnSOD, GPX, and Catalase were evaluated with Western blot analysis.

Results: Maternal plasma ADMA concentrations were elevated in the first trimester of pregnancy and remained elevated at delivery in women with SGA (P<0.05, respectively). We detected higher total eNOS expression and lower eNOS phosphorylation at serine^{1177} in women with SGA compared with women with uncomplicated pregnancies (P<0.05). Maternal plasma GPX activities in the first trimester of pregnancy and the placental MnSOD, GPX, and Catalase expression were not significantly different between women with SGA and women with uncomplicated pregnancies (P>0.05, respectively).

Conclusions: We suggest that women with elevated ADMA concentrations at the first trimester have a higher risk of experiencing pregnancies with SGA. Furthermore, the results of this study demonstrate that the mechanism for SGA may not be altered antioxidant enzyme activity in placenta but another mechanism.

Key words: ADMA, SGA, eNOS, Placenta, Antioxidant enzyme

INTRODUCTION

Fetal growth restriction (FGR) is diagnosed when the fetus is estimated to be too small for gestational age (SGA), and SGA also has fetal morbidity and more severe neonatal complications. Although reduced placental perfusion and endothelial dysfunction appear to be important factors relating to the SGA, the pathogenesis of SGA is unclear.[1]

Nitric oxide is synthesized from L-arginine in the vascular endo-

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thelial cells. It induces vessel dilation and inhibits the activation of platelets, leukocyte adhesion, smooth muscle proliferation, etc. NO is synthesized from L-arginine via the activity of NO synthase (NOS). NOS is partially regulated by negative feedback from NO, but there are also other important inhibitors present endogenously in humans, such as the competitive inhibitor asymmetric dimethylarginine (ADMA).[2] Nitric oxide acts as an antioxidant to decrease superoxide radical formation in the vessel, and decreases the oxidation of LDL cholesterol.[3,4] If the plasma ADMA level increases, the generation of nitric oxide decreases, enhancing oxidative stress and decreased antioxidant. There were some conflicting results regarding ADMA concentration in pregnancies with SGA. Savvidou et al. reported that elevated ADMA concentrations at 23-25 weeks' gestation preceded pregnancies with FGR with abnormal uterine artery Doppler in the absence of preeclampsia.[5] However, according to Speer et al, maternal ADMA concentrations were higher in mid-pregnancy in women who experienced preeclampsia and not in women who experienced SGA.[6]

To explain the pathogenesis of pregnancies with SGA, the placenta is a key, and the main hypothesis might involve oxidative stress. An imbalance between prooxidants (reactive oxygen species) and antioxidants results in oxidative stress. Protein- based antioxidant enzymes include Manganese Superoxide Dismutase (MnSOD), Glutathione Peroxidase (GPX), and Catalase. There have been some controversial results about placental eNOS expression in women with SGA and an evidence for placental antioxidant enzyme activity in women with FGR.[7-10] Casanello et al. demonstrated that human umbilical vein endothelial cells from pregnancies with SGA exhibited lower eNOS expression and phosphorylation.[11] The objective of this study was to evaluate whether maternal plasma concentrations of ADMA in the first trimester of pregnancy and at the time of delivery in women with SGA are increased and to demonstrate whether maternal plasma GPX activities in the first trimester of pregnancy, placental expression of total eNOS, eNOS phosphorylation at serine^{1177}, and antioxidant enzymes are changed in women with SGA, compared with women with uncomplicated pregnancies.

MATERIAL AND METHODS

1. Study population

This study was a nested case-control study from Ewha Womans University Hospital in Seoul, Korea. The study was approved by the Institutional Review Board and informed consent was obtained from all subjects. Subjects were recruited to the study at the time of admission to Ewha Womans University Hospital for prenatal care.

Four hundred pregnant women were recruited to study at the time of admission to Ewha Womans University Hospital for prenatal care. All subjects, including cases and controls, were healthy women without known medical complications. After delivery, the medical records of the subjects were reviewed by a physician. Subjects were matched for parity, maternal age, and gestational weeks at delivery. Among 400 pregnant women, uncomplicated control pregnancies (n=20) and subjects with SGA (without preeclampsia) (n=20) were chosen. SGA was defined by infant birth weight below the tenth percentile, after adjustment for gestational age and gender, in an otherwise uncomplicated pregnancy.[12] SGA infants with clinical or pathologic evidence of infection or congenital or chromosomal abnormalities or preeclampsia were excluded from the study.

2. Blood samples

Maternal venous EDTA plasma samples were collected between 10-13 weeks' gestation during prenatal visits and again at the time of admission for delivery. Samples were stored at -70^{o}C for analysis. These samples were part of a collection that was accumulated longitudinally. The average storage time was 5.6 years. The mode of delivery was not significantly different among the study groups (P>0.05).

3. Measurement of ADMA

The measurement of plasma ADMA concentration was performed

by a quantitative enzyme linked immunoassay (ELISA) using a commercial kit (Immundiagnostik AG, Germany) according to manufacturer's instructions. Briefly, plasma samples were mixed with reaction buffer and acylation buffer followed by the addition of samples into microtiter plate wells. After adding the ADMA antibody as well as peroxidase conjugated antibody into each well, TMB substrate was added for color development. Optical density was measured at 450 nm against 620 nm as a reference using a spectrophotometric reader (VersaMax, Molecular devices, Sunnyvale, CA). The intra-assay coefficient of variations (CVs) for ADMA-ELISA were 10% for kit control 1 (0.88±0.088 μmol [μM]) and 2.5% (1.74±0.044 μM) for kit control 2. Inter- assay CVs for ADMA-ELISA were 7.5% for kit control (0.84± 0.063 μM) and 5.5% for kit control 2 (1.78±0.098 μM). Detection limit was 0.05 μM.

4. Measurement of GPX activity

GPX activity measurement was based on the following principle: GPX catalyzes the oxidation of reduced gluthathione by tertiary bytul hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidized gluthathione is converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. Plasma was mixed with gluthathione, gluthathione reductase and 0.1 M phosphate buffer contaning H_2O_2, and incubated for 10 min at 37°C. NADPH and tertiary bytul hydroperoxide were added and the oxidation of NADPH was monitored at 340 nm for 5 min using spectrophotometry. The GPX activity of plasma was expressed as international units per weight (g) protein.[13]

5. Western blotting

After delivery, all of the layers placentas were excised, and the collected samples were frozen in refrigerator at -70°C. The placenta was homogenized with tissue homogenizer after adding lysis buffer including 1.0 mM phenylmethylsulfonyl fluoride, 1.0 mM EDTA, 1 mM pepstatin A, 1 mM leupeptin, and 1 mM aprotinin (Intron Biotechnology, Seoul, Korea). The lysate was analyzed for its protein content using a Bicinchoninic Acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL). Equal amounts of protein were resolved under reducing conditions on a 10% of SDS-polyacrylamide gel and transferred into a polyvinylidene difluoride membrane (PVDF). Western blotting was performed using an antibody for mouse-antihuman monoclonal total eNOS (BD Bioscience pharmingen, San Jose, CA), mouse-antihuman phosphorylated ^{1177}Ser-eNOS (BD Bioscience, San Jose, CA), mouse-antihuman monoclonal MnSOD (BD Bioscience pharmingen, San Jose, CA), rabbit-antihuman monoclonal GPX (Young In Frontier, Seoul, Korea), or rabbit-antihuman monoclonal catalase (Young In Frontier, Seoul, Korea). The membranes were then probed with the respective secondary antibodies conjugated to horseradish peroxidase (Southern Biotech, Birmingham, AL). After that, the PVDF membrane was exposed on X-ray film (Kodak, New York, NY). Densitometry was performed with gel documentation (Gel Doc 2000 Quantity One; Bio-Rad, Hercules, CA), and the results were expressed by densitometry units (INT/mm^2).

6. Statistical analysis

Power analysis using Study Size 2.0.4 Trial version indicated that we would require at least 20 subjects in each study group to achieve an alpha of 0.05 and at least 80% power.[5] For statistical analysis of the study results, Statistical Package for Social Sciences (PC Windows version 8; SPSS, Chicago, IL) was used. The differences among the study groups were analyzed by the Mann- Whitney U test and Wilcoxon rank sum test with a cutoff of $P<0.05$.

RESULTS

The clinical characteristics of the study groups are shown in Table 1. There were no significant differences in maternal age and gestational age at delivery between the study subjects ($P>0.05$). However, birth weight was significantly lower in women with SGA pregnancies ($P<0.05$). There were no significant differences in the

Table 1. Clinical characteristics of control and pregnancy with SGA

	Control (n=20)	SGA (n=20)
Age	31.30±2.71	32.10±4.36
Gestational weeks (weeks)	37.63±0.39	36.48±2.53
Birth weight (g)	3235.0±310.1* (80 percentile)	2040.0±514.9† (5 percentile)

Values are given as Mean±SD and statistical significance was assessed by the Mann-Whitney U test.
SGA: small for gestational age.
* vs †, $P<0.05$.

Table 2. Plasma ADMA levels in the 1st trimester and at delivery between control and pregnancy with SGA

	Control (n=20)	SGA (n=20)
Plasma ADMA in the 1st trimester (μM/L)	0.56 (0.35-1.72)*	0.85 (0.55-1.23)†
Plasma ADMA at delivery (μM/L)	1.08 (0.82-1.41)‡	1.34 (1.02-1.90)§

Values are given as Median (IQR) and statistical significance was assessed by the Mann-Whitney U test.
ADMA: asymmetric dimethylarginine, SGA: small for gestational age.
* vs †, $P<0.05$, ‡ vs §, $P<0.05$.

Table 3. Density of placental MnSOD, GPX, and catalase by western blot in control and pregnancy with SGA

	Control (n=20)	SGA (n=20)
MnSOD (INT/mm^2)	0.84 (0.59-1.29)	1.11 (0.55-1.59)
GPX (INT/mm^2)	0.30 (0.25-0.42)	0.23 (0.16-0.41)
Catalase (INT/mm^2)	1.38 (0.92-1.62)	1.23 (0.97-1.87)

Values are given as Median (IQR) and statistical significance was assessed by the Mann-Whitney U test.
SGA: small for gestational age, MnSOD: manganese superoxide dismutase, GPX: glutathione peroxidase.
$P>0.05$.

proportion of smoking subjects among study groups and no differences between groups in the sample collection time at first trimester of pregnancy (data is not shown, $P>0.05$). The mode of delivery was not also significantly different among the study groups (data is not shown, $P>0.05$).

In the samples that were collected during the first trimester of pregnancy, mean maternal plasma ADMA concentrations were significantly higher in women who later experienced SGA pregnancies, compared with uncomplicated pregnancies ($P<0.05$) (Table 2). At delivery, plasma ADMA concentrations were still significantly higher in women with SGA pregnancies, compared with uncomplicated pregnancies ($P<0.05$, respectively) (Table 2).

There were no significant differences in maternal plasma GPX activity during the first trimester of pregnancy (2545.0±1342.0 units/g for SGA pregnancy vs 2545.0±1342.0 units/g for uncomplicated pregnancy). In the Table 3, there were no significances of placental MnSOD, GPX, and Catalase expression between SGA pregnancies and uncomplicated pregnancies ($P>0.05$, respectively) (Table 3). In placental total eNOS and phosphorylated ^{1177}Ser-eNOS expression, we detected higher total eNOS and lower eNOS phosphorylation at serine^{1177} expression in women with pregnancies with SGA compared with women with uncomplicated pregnancies ($P=0.03$, $P=0.01$, respectively) (Fig. 1).

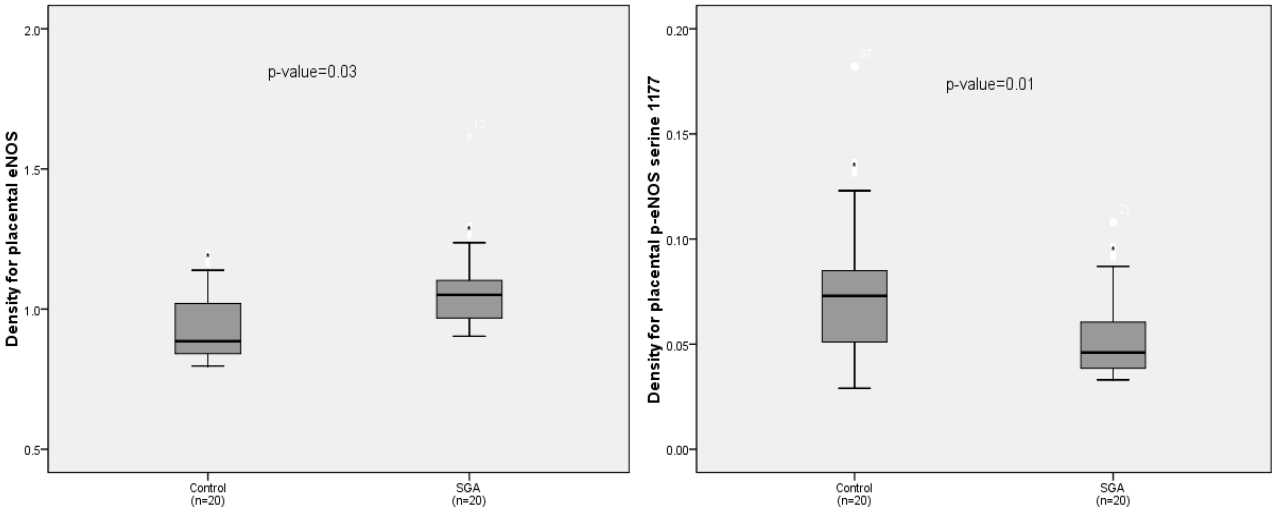

Fig. 1. Distribution of total eNOS and eNOS phosphorylation at serine^{1177} expression in placentas of control and pregnancy with SGA. The median line of the box indicates the middle 50% of the data and the upper and lower hinges of the box represent the 75th and 25th percentiles of the data, respectively. The lower and upper lines represent the minimum and maximum values, respectively. Statistical significance was assessed using the Wilcoxon rank sum test.
eNOS: endothelial nitric oxide, SGA: small for gestational age.

DISCUSSION

In this study, we observed that the concentration of maternal plasma ADMA during the first trimester of pregnancy is significantly higher in women who later experience pregnancies with SGA than in women with uncomplicated pregnancies, and this difference continues at delivery time. In our experience, this is the first evidence reporting that elevated ADMA concentrations during the first trimester precede pregnancies with SGA and that maternal plasma ADMA concentrations could be useful as an early marker during the first trimester of pregnancy to detect pregnancies with SGA.

Only two papers have considered the relationship between pregnancies with SGA and maternal plasma ADMA concentrations.[5,6] According to Savvidou et al., higher maternal plasma ADMA concentrations at 23-25 weeks' gestation in women with an abnormal uterine artery Doppler was associated with pregnancies with SGA.[5] On the other hand, the study by Speer et al. showed that maternal ADMA concentrations were not higher in mid-pregnancy in women with SGA.[6] To explain the difference between the study of Savvidou et al. and the study of Speer et al., the authors of the latter study pointed out the severity of growth-restricted infants and methodological differences between two studies.[6] However, our study showed the same results as the study of Savvidou et al., even though we measured maternal plasma ADMA concentrations at 10-13 weeks of gestation. In the aspect of methodological difference, our study used ELISA method and other studies including Savvidou et al and Speer et al used HPLC method. The concentration of plasma ADMA in the study of Savvidou et al[5] (average ADMA concentration, 0.81 μmol/L) was much higher than that of Speer et al[6] (average ADMA concentration, 0.34 μmol/L). However, the concentrations of plasma ADMA in our study were 0.56 μmol/L at first trimester and 1.08 μmol/L at delivery and this concentration was higher than that of Speer et al and lower than that of Savvidou et al.[5,6]

The present study showed up-regulated total eNOS expression and down-regulated eNOS phosphorylation at serine^{1177} in women with pregnancies with SGA. Although conflicting results have been reported with regard to total eNOS expression in women with pregnancies with SGA, our evidence for eNOS phosphor-

ylation at serine[1177] using placenta with SGA pregnancies is the first evidence. According to Schiessl et al using histochemistry and western blot, the expression of total eNOS was significantly reduced in trophoblast cells of placentas with SGA.[8] Casanello et al. demonstrated that human umbilical vein endothelial cells from SGA pregnancies exhibited lower eNOS expression and phosphorylation.[11] However, Myatt et al. using immunohistochemistry demonstrated that the expression of eNOS in the placentas of women with SGA pregnancies was increased.[9] Giannubilo et al. using mRNA expression and immunohistochemistry observed that no difference in total eNOS mRNA expression in the placenta of SGA was found, although they observed a significant elevation of inducible NOS (iNOS).[14] The explanation for up-regulated expression of placental total eNOS expression in the placenta of SGA pregnancy may be an adaptive physiological mechanism to overcome a deficiency in the fetoplacental circulation in pregnancies complicated by SGA.[14] Our result showed a different result with Schiessl et al.[8] Schiessl et al showed reduced expression of total eNOS and this difference between ours and Schiessl et al may be due to the severity of SGA fetus. Our results also showed up-regulated expression of placental total eNOS through a compensatory mechanism. However, the fact of lower eNOS phosphorylation at serine[1177] in SGA placenta demonstrates that SGA placenta has reduced NO synthesis and increased ADMA via decreased eNOS phosphorylation.[11]

In evaluating the placental expression of antioxidant enzymes, we found that the placental MnSOD, GPX, and Catalase expression were not significantly different between women with SGA and women with uncomplicated pregnancies. According to Biri et al., placental tissue SOD activity was not significantly different and placental tissue GPX activity was significantly higher in women with SGA.[10] However, placental catalase activity was decreased in women with SGA.[10] Regarding these findings, they explained that the SOD activity, which increases with oxidative stress, might induce GPX activity and the catalase activity might decrease as a compensation mechanism.[10] Although Biri et al. measured placental antioxidant enzyme activity and we measured placental antioxidant enzyme expression by Western blot, the results showed different findings. The cause showing different results between Biri et al. and ours would be the difference of gestational weeks. In case of Biri et al., the gestational weeks in SGA groups was 33-34 weeks and that of our groups was 36-38weeks. Because the gestational weeks in Biri et al. was 33-34 weeks and the cases were preterm delivery, higher level of oxidative stress in SGA pregnancy would be attributed by preterm delivery, not by SGA pregnancy.[15] In our study, we also measured maternal plasma GPX activity during first trimester and observed that no difference between women with SGA and women with uncomplicated pregnancies was found.

From our findings, we can consider that there are another explanations in the mechanism of SGA pregnancy other than oxidative stress. For the mechanism of unexplained SGA, Yung et al. suggested the first evidence placental synthesis inhibition and endoplasmic reticulum stress.[16] Additional studies are needed to investigate the mechanism of SGA pregnancies. Although this is the first evidence reporting that elevated ADMA concentrations during the first trimester precede pregnancies with SGA and that maternal plasma ADMA concentrations could be useful as an early marker during the first trimester of pregnancies preceding SGA pregnancies, we also have a limitation because we didn't measure the oxidative stress marker and NO level in maternal plasma samples.

In conclusion, we suggest that women with elevated ADMA concentrations during the first trimester have a higher risk of pregnancies with SGA and elevated ADMA levels are resulted by decreased placental eNOS phosphorylation.

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「국문초록」

목적: 본 연구는 저체중아 임신에서 임신 제1분기의 asymmetric dimethylarginine (ADMA)과 glutathione peroxidase activity (GPX)의 혈장내 농도의 변화를 알아보고 태반내 endothelial nitric oxide synthase (eNOS)와 antioxidant enzyme의 발현을 알아보고자 하였다.

연구방법: 정상 임신부와 저체중아 임신한 여성에게서 임신 10-13주와 임신 말기에 각각 혈액을 수집하고 혈장내 ADMA는 ELISA로 측정하고 GPX는 spectrophotometry로 측정하였다. 태반내 eNOS, eNOS phosphorylation, MnSOD, GPX,및 Catalase는 western방법으로 측정하였다.

결과: 저체중 임신의 경우 임신부의 혈장내 ADMA는 임신 초기와 분만 시에 증가되어 있었으며 저체중 임신의 경우 태반내 eNOS의 발현은 증가되어 있었고 eNOS phosphorylation의 발현은 감소되어 있었다. 임신 초기 혈장내 GPX activity는 정상 임신부와 저체중 임신부에서 차이가 없었으며 태반내 항산화 효소의 발현에도 변화가 없었다.

결론: 이상의 결과로 임신부에서 임신 초기의 ADMA는 저체중아 임신의 가능성을 증가시키며 이러한 저체중아 임신에 있어서 항산화 효소의 발현에 변화가 없으므로 다른 기전이 관여할 것으로 사료된다.

중심 단어: ADMA, 저체중아 임신, eNOS, 태반, 항산화 효소