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Placental Endothelial Nitric Oxide Synthase in Multiple and Single Dose Betamethasone Exposed Pregnancies

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Abstract

Objective—To compare endothelial nitric oxide synthase (eNOS) expression and capillary density (CDS) in placentas exposed to single or multiple courses of betamethasone.

Study design—Placental specimens exposed to single versus repeat courses of betamethasone were analyzed through immunohistochemistry and digital image quantification for eNOS and CD34. Quantified eNOS staining, calculated CDS, ratio of eNOS to CDS, and clinical characteristics were compared. Linear regression performed with these as dependent variables.

Results—Mean and maximum CDS were increased ($p=0.013$ and 0.005) and the ratio of eNOS to CDS decreased ($p=0.016$) in specimens exposed to 4 courses of betamethasone compared with 1 to 3 courses. Exposure to 4 courses of betamethasone was associated with increased CDS, but not with eNOS expression.

Conclusion—Exposure to 4 courses of betamethasone is associated with increased placental CDS. The placental effects of multiple courses of betamethasone are unrelated to eNOS expression.

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Keywords

betamethasone; eNOS; placenta

INTRODUCTION

Multiple courses of antenatal betamethasone have been shown to result in decreased birthweight and neonatal head circumference.^{1,2} However, the mechanism of this effect remains unclear.

In studies examining fetal growth restriction, it has been found that the large increase in fetal growth during the last half of pregnancy depends primarily on a corresponding increase in transplacental exchange.^{3,4} This occurs through coordination of angiogenesis, adaptations of vascular tone, and efficiency of exchange and is supported by significant growth of the placental vascular beds with resultant increases in uterine and umbilical blood flow.^{5,6} It has further been shown that the vascular density of fetal placental components increases dramatically during the last third of gestation as compared with maternal components which have been shown to have a consistent slow increase throughout gestation.^{6,7,8}

Endothelial nitric oxide synthase (eNOS) is an important component of regulation of vascular tone and blood flow and has been linked to feto-placental growth and development. Several authors have postulated that this is a result of eNOS mediated nitric oxide release from the fetal tissues and is supported by immunoreactive and in situ hybridization demonstration of eNOS expression by extravillous trophoblast cells.^{9,10}

Additionally, antenatal glucocorticoid exposure in primates has been shown to significantly reduce eNOS protein as well as other indices of eNOS function. This effect has been seen in both the syncytiotrophoblast as well as the vascular endothelium.^{9,11}

The objective of this study was to compare eNOS expression and capillary density in placentas from pregnancies exposed to multiple or single courses of betamethasone. It was our hypothesis that betamethasone exposure influences fetal growth through a nitric oxide mediated mechanism.

MATERIALS AND METHODS

Study design

This study is an analysis of placental specimens collected during the conduct of a randomized multicenter trial that compared the effect of administering single versus weekly repeat courses of antenatal betamethasone in preventing neonatal morbidity. The trial was conducted between March 2000 and April 2003 at participating centers of the the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, Maternal-Fetal Medicine Units (MFMU) Network. The primary study has been previously described.¹ Participants in the trial were pregnant women between 23 weeks 0 days and 31 weeks 6 days, who were at risk for spontaneous preterm delivery. Women with preterm premature rupture of the membranes (PPROM) prior to randomization, confirmed fetal lung maturity, chorioamnionitis, a major fetal anomaly, non-reassuring fetal status, systemic corticosteroid use during the current pregnancy, or insulin-dependent diabetes were excluded.

Patients were randomized to receive weekly courses of betamethasone or placebo, one week after receiving a single full course of corticosteroids (betamethasone or dexamethasone).

Each course consisted of two injections of betamethasone 12 mg (as 6 mg betamethasone sodium phosphate and 6 mg betamethasone acetate) repeated once in 24 hours or matching placebo. Initially, patients received courses until delivery or 33 weeks 6 days' gestation, whichever was sooner. After 67 patients had been enrolled, the number of courses (not including the qualifying course) was limited to 4 because of difficulty in recruitment and because of accumulating literature suggesting possible harmful effects of multiple courses.

Placentas were collected at the time of delivery. For this analysis, placental specimens from patients with singleton pregnancies were evaluated.

Placental specimen preparation

Specific samples were obtained from each placental specimen and were formalin fixed and paraffin embedded. All samples included in this analysis were from a standardized lateral location within the placenta containing both maternal and fetal surfaces. Institutional Review Board approval was obtained before conducting this investigation.

After obtaining the paraffin blocks of these placental tissues, sections were cut at four microns, adhered to ProbeOn Plus slides from a protein-free water bath and air-dried overnight. Slides were then deparaffinized and hydrated to deionized water. Following HIER (Heat Induced Epitope Retrieval) in Citrate buffer pH 6.0 for 30 minutes using a Black and Decker rice steamer (eNOS/NOS type III, BD Transduction Laboratories) and enzymatic retrieval using Trypsin at 1 mg/ml, 4mM CaCl₂ in 20mM TRIS pH 7.7 for 30 minutes at 37°C, slides were washed for five minutes under running deionized water, then transferred to a hand-held MicroProbe slide holder. A 1X Automation Buffer (Biomeda) containing 0.1% Tween 20 to eliminate surface tension and 0.5% Casein (Sigma) to blocking non-specific background, was used both as wash buffer and antibody diluent. Serial sections were incubated with mouse monoclonal eNOS/NOS Type III (BioGenex) at 1:50 and monoclonal CD34 (Novocastra) at 1:10 overnight at 4°C. Following several washes with 1X buffer, slides were incubated with biotinylated anti-mouse antibodies (BioGenex) at 1:20 for 20 minutes at 37°C. Following additional washes using 1X buffer, sections were incubated with a Streptavidin-alkaline phosphatase complex (BioGenex) at 1:20 for 20 minutes at 37°C. The red end product was visualized using a Vector Red chromogen (Vector Laboratories). Slides were counterstained for five minutes in Mayer's hematoxylin, washed under running deionized water, dehydrated using graded alcohols, cleared through p-xylene and mounted with Permount (Figure 1, Figure 2). This technique is applied routinely to paraffin embedded specimens in both clinical and research immunohistochemistry laboratories and the antibodies employed in this investigation were specifically noted for use on paraffin sections.¹² The HIER process described above enhances this technique and consists of enzymatic retrieval of protein cross linkages that might have occurred through formalin fixation in these tissues, allowing return to a native configuration of any proteins that may have been altered during fixation.¹³

Digital images of each slide were then created and staining was quantified for both eNOS and CD34 using Image Pro Plus software. This quantification was based on the number of pixels in each digital image exhibiting immunostaining (Figure 3). Each micrometer stained represented 2,160 pixels in this model. The slides were analyzed in quadrants with measurements taken from all four quadrants for each specimen. A mean was also calculated for each specimen from these four measurements.

Capillary area density, defined as the CD34 staining area as a proportion of total field area, was then calculated for each specimen. Total field area for the slides employed in this study was found to be 76,3202.1 micrometers. This is a well accepted approach that has been used to quantitate angiogenesis in placental tissues. This has also been correlated with expression

of angiogenic factors.^{14,15,16} An additional parameter, the ratio of eNOS to capillary area density was calculated for each specimen utilizing mean eNOS staining and mean capillary area density values. All investigators remained blinded to the clinical group assignment of the specimens until all immunohistochemistry and staining quantification had been completed.

Statistical analysis

The main outcomes for this analysis were capillary area density, eNOS expression, and the ratio of eNOS to capillary area density. Treatment and placebo groups were compared. Patients in each study arm who received one to three study courses versus four study courses were also examined. Chi-square or Fisher's Exact test was used to compare categorical variables and the Wilcoxon Rank Sum test was used to compare continuous variables. Linear regression models in which the effect of treatment group exposure to four study courses and gestational age at delivery with the outcomes of capillary area density and eNOS were performed.

Additional regression analysis of patients assigned to the active group which examined the effect of exposure to 4 study courses versus one to three courses was also performed. Possible confounding variables included in these models were maternal age, parity, ethnicity, maternal body mass index, maternal level of education, gestational age at randomization, gestational age at delivery, tobacco use, presence of preterm labor, exposure to tocolytics, maternal infection, preterm premature rupture of membranes, and presence of placental calcification and placental infarction recorded as present or absent in the initial pathology exams of these placentas.

A nominal P value of less than 0.05 was considered to indicate statistical significance, and no adjustments were made for multiple comparisons.

RESULTS

There were 191 placental specimens able to be completely analyzed. The active treatment group, receiving multiple courses of betamethasone, represented 100 and the placebo group, receiving a single course of betamethasone, represented 91 of these samples. Of those in the active treatment group, 68 had exposure to 4 courses of antenatal betamethasone, 32 had exposure to 1 to 3 study courses. Baseline clinical characteristics for patients receiving single and multiple courses of betamethasone were similar (Table 1).

There were no significant differences in the mean or maximum capillary area density or in the mean and maximum quantified eNOS expression between the active and the placebo groups (Table 2). When results were stratified by number of study courses, mean and maximum capillary area density were increased (Table 3) and the ratio of eNOS to capillary area density was decreased (Table 3), in patients in the active group exposed to 4 study courses compared with those who received 1 to 3 courses. Mean and maximum eNOS staining was not significantly different between these groups.

The association of treatment group, exposure to four study courses, and gestational age at delivery with the outcomes eNOS, capillary density, and ratio of eNOS to capillary density was examined using linear regression analysis. There was a significant association between the interaction of treatment group and exposure to four study courses and capillary density ($p=0.047$). Gestational age at delivery was directly associated with capillary density ($p=0.0025$) and inversely associated with eNOS expression ($p=0.031$) and the ratio of eNOS expression to capillary density ($p=0.0009$).

Regression analysis was performed for those who received four study courses, either active or placebo which adjusted for possible confounding factors including maternal age, parity, ethnicity, maternal body mass index, maternal level of education, gestational age at randomization, gestational age at delivery, tobacco use, presence of preterm labor, exposure to tocolytics, maternal infection, preterm premature rupture of membranes, and presence of placental calcification and placental infarction. This analysis showed that exposure to four courses of betamethasone was significantly associated with increased capillary density ($p=0.018$) compared with receipt of a single course. Capillary density was also significantly associated with gestational age at randomization ($p=0.004$) and maternal age ($p=0.015$).

Further regression analysis of the active treatment group which adjusted for the same possible confounding factors listed above showed that exposure to four courses of betamethasone was significantly associated with increased capillary density ($p=0.0075$) compared with 1 to 3 courses. Increasing maternal age was also associated with increased capillary density ($p=0.021$).

COMMENT

This investigation examines human placental eNOS in the setting of a clinical trial comparing single to multiple courses of betamethasone. The rigorous study design of the clinical trial preceding this investigation and the volume of samples included are clear strengths of this analysis.

Our findings likely indicate that the fetoplacental effects of antenatal betamethasone exposure occurs independent of the influences of eNOS expression. We did not find differences in mean and maximum eNOS staining in those placentas exposed to single as compared with multiple antenatal courses of betamethasone. This finding persisted even when results were stratified according to specific number of betamethasone courses.

In pregnancies exposed to 4 antenatal betamethasone courses, mean and maximum capillary density was increased as compared to those exposed to 1 to 3 courses. Further, the ratio of eNOS to capillary density decreased in these specimens, confirming the unlikely influence of eNOS in these processes. This suggests that there may be other regulators of angiogenesis that are affected by antenatal betamethasone exposure to four courses of antenatal betamethasone were the ones found to have the most pronounced effects on fetal growth (1). These data support a nitric oxide independent compensatory mechanism to stimulate capillary growth when the fetoplacental unit is exposed to multiple courses of antenatal betamethasone or other similar insults.

However, despite increased capillary density, pregnancies exposed to 4 courses of antenatal betamethasone were the ones found to have the most pronounced effects on fetal growth.¹ This suggests an angiogenic response to repeated betamethasone exposure that is not able to improve exchange within the fetoplacental unit and questions the functional integrity of these vessels.

We also found that increasing gestational age was associated with decreasing placental eNOS expression and increasing capillary density. These findings have been suggested in other similar investigations in mammalian species.^{17,18} Our study has uniquely focused on examining eNOS as it is related to capillary density in human placentas.

Additionally, we found that increasing maternal age was associated with increased placental capillary density. The clinical significance of this is uncertain at the present time but again suggests that there may be other important compensatory mechanisms in place that influence placental angiogenesis in the particular circumstance of increasing maternal age and

exposure to multiple antenatal courses of betamethasone. The question also arises as to whether the effects of maternal age on the fetoplacental unit may be similar to those of exposure to multiple antenatal courses of betamethasone. This is supported by recent reports of an association between advancing maternal age and poor pregnancy outcomes, including fetal growth restriction.^{19,20}

This study has several limitations. The most significant of which is that we do not have data from these specimens that includes Western blotting. Thus, we are unable to make comparisons between our immunohistochemical staining and the results of Western blotting in these specimens. Additionally, regional differences may exist within the placenta. We have studied a single lateral placental location in these specimens and while this was standardized for every specimen, it could be postulated that differences in other locations within the placental bed may exist.

The regulation and influence of eNOS is complex and immunohistochemistry at a single clinical point in time may not be able to adequately account for variations through gestation. It is likely that placental eNOS may have influences that precede the findings at delivery and that are more pronounced earlier in gestation. Our results support this as eNOS expression in these specimens was inversely related to gestational age. The results of other investigators confirm this relationship as well as the complex interactions of eNOS within the maternal and fetal compartments.^{21,22}

In summary, this investigation provides novel information regarding the placental effects of antenatal exposure to multiple as compared with single courses of betamethasone. While eNOS does not appear to be involved in these effects, there does seem to be an important influence on placental angiogenesis in the setting of exposure to four courses of antenatal betamethasone. Ongoing investigation is needed to more clearly elucidate the mechanisms of the effects of antenatal betamethasone exposure on the fetoplacental unit. Such investigations have the potential to give further insight into the mechanisms of the effects of betamethasone exposure during pregnancy as well as normal and abnormal fetal growth.

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Appendix

In addition to the authors, other members of the *Eunice Kennedy Shriver National Institute of Child Health and Human Development Maternal-Fetal Medicine Units Network* are as follows:

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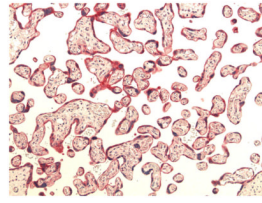


Figure 1.
Immunohistochemistry staining for eNOS in human placental tissue.

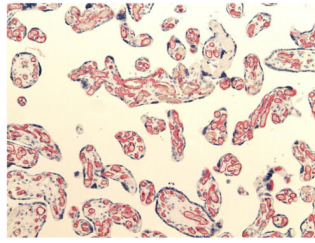


Figure 2. Immunohistochemistry staining for CD34, an endothelial vascular stem cell antigen in human placental tissue.

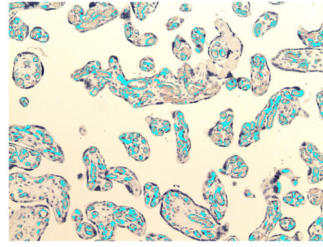


Figure 3.
Digital image quantification of CD34 immunostaining in human placental tissue.

Table 1

Clinical characteristics of patients receiving single and multiple courses of antenatal betamethasone.

Group	Single course N=91	Multiple courses N=100	p-value
Maternal age (years) (mean \pm SD)	25.9 (5.9)	25.9 (5.8)	1.0
Ethnicity (number (%))			0.73
African American	38 (41.8)	37 (37.0)	
Caucasian	29 (31.9)	37 (37.0)	
Hispanic/other	24 (26.4)	26 (26.0)	
Maternal body mass index (mean \pm SD)	26.4 (7.0)	26.1 (6.3)	0.94
Gestational age at randomization (weeks) (mean \pm SD)	28.2 (2.4)	27.9 (2.4)	0.40
Gestational age at delivery (weeks) (mean \pm SD)	35.2 (4.1)	35.0 (3.8)	0.71
Tobacco use (number [%])	23 (25.3)	27 (27.0)	0.79
Nulliparous (number [%])	8 (8.8)	10 (10.0)	0.78
Preterm labor/treatment with tocolytics (number [%])	53 (58.2)	53 (53.0)	0.47
Maternal infection(number [%])	31 (34.1)	26 (26.0)	0.22
Preterm premature rupture of membranes (number [%])	14 (15.4)	15 (15.0)	0.94
Chorioamnionitis (number [%])	3 (3.3)	3 (3.0)	1.0000
Abruption (number [%])	8 (8.9)	8 (8.8)	0.83

Table 2

Capillary area density and quantified eNOS expression in placentas of patients exposed to single and multiple courses of antenatal steroids

Group	Single course N=91	Multiple courses N=100	p-value
Maximum capillary density (mean \pm SD)	7475.86 (2340.10)	7441.20 (2048.16)	0.72
Maximum eNOS staining (mean \pm SD)	13258.14 (3247.17)	13154.66 (3487.22)	0.84
Mean capillary density (mean \pm SD)	5807.09 (1722.63)	5820.92 (1655.43)	0.73
Mean eNOS staining (mean \pm SD)	11028.49 (2528.29)	10903.94 (2658.54)	0.76
Ratio of eNOS to capillary density (mean \pm SD)	2.08 (0.82)	2.05 (0.82)	0.74

Table 3

Capillary area density and quantified eNOS expression for patients exposed to four courses versus one to three study courses of betamethasone in the active group.

Group	1 to 3 Courses N=32	4 Courses N=68	p-value
Maximum capillary density (mean \pm SD)	6715.4 (1784.5)	7782.7 (2086.9)	0.005
Maximum eNOS staining (mean \pm SD)	13571.2 (3361.7)	12955.7 (3553.1)	0.27
Mean capillary density (mean \pm SD)	5244.3 (1487.4)	6092.3 (1671.0)	0.013
Mean eNOS staining (mean \pm SD)	11293.4 (2652.2)	10717.9 (2661.2)	0.21
Ratio of eNOS to capillary density (mean \pm SD)	2.33 (0.84)	1.92 (0.78)	0.02