Nitrous Oxide Synthase Expression in Placenta of Preeclampsia

To compare the expression of endothelial nitrous oxide synthase (e-NOS), which plays a major role in vasoconstriction, in preeclampsia with that in the normotensive placenta and to identify whether or not e-NOS actually influences the pathogenesis of preeclampsia, we have studied the distribution and intensity of the endothelial isoform of NOS expression in preeclampsia (n=121) and normal (n=14) pregnancies. Archival paraffin-embedded tissues were immunostained with a polyclonal e-NOS antibody. E-NOS was stained predominantly in the endothelial cells of the umbilical cord and along the surface of the villi, especially within the cytoplasms of the syncytiotrophoblasts with a granular and punctuated pattern, but not in the trophoblastic island or in the intravillous capillaries. The degree of e-NOS expression in pregnancy-induced hypertension (PIH) was significantly lower than in normotensive placenta, and moreover in the patients lacking abruptio. The expression of e-NOS in preeclampsia has some correlation with patient age. In conclusion, the degree of e-NOS expression in cotyledon from PIH is far less than from control, it means a kind of hemodynamic alteration of the fetoplacental circulation in preeclampsia.
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Key Words : *Nitrous oxide; Pre-eclampsia; Immunohistochemistry; Placenta*

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INTRODUCTION

Preeclampsia, an exclusively gravid syndrome unique to humans, involves many organs where endothelium may be altered (1-3). The etiology of preeclampsia remains unclear, the fact has been widely accepted that many of its pathophysiologic changes are essentially a failure of the compensatory responses described in normal pregnancy (1, 4). Not surprisingly, numerous studies have shown elevated systemic vascular resistance in pregnancy-induced hypertension (PIH) when compared to normal pregnancy (5-8). In contrast to a normal pregnancy focusing on high volume, low pressure and low resistance, hemodynamic studies in PIH may be described as low-volume, high-pressure and high-resistance (1-9). This underlying processes that culminate in the clinical manifestations of PIH are poorly understood. It has been proposed that widespread endothelial dysfunction, resulting in vasospasm, altered vascular permeability and activation of coagulation could lead to clinical findings observed in PIH women (4-10). The precise factors leading to vasospasm and sensitivity to pressor substance, such as angiotensin II, has been the prime focus of PIH research for many years (1). Recently, endothelin, a novel hormone functioning as a vasoconstrictor, has been suspected of playing a leading role in vasospasm (11). Now, current evidences with respect to the role of endothelium-derived relaxing factor (EDRF), nitrous oxide radical (NO), have shown up (12-13). NO radical appears to play a major role in the regulation of maternal and fetoplacental hemodynamics during pregnancy. NO was released from endothelial cells by bradykinin in amounts that accounted for the action of EDRF, a conclusion that was quickly confirmed (14). NO with prostacyclin are potent vasodilators and can also act separately or synergistically to prevent adhesion and aggregation of platelets (15). At least 3 isoforms of NO synthase enzyme (NOS) have been cloned, one from rat cerebellum (b-NOS), one from human and bovine endothelial cells (e-NOS), and one from mouse macrophage (m-NOS) (12-16). These isoforms bear sequence homology to each other, but whereas the e-NOS and b-NOS are constitutively expressed and require calcium-calmodulin for maximal activity (17), the activity of m-NOS is dependent on de novo RNA and protein synthesis induced by a variety of cytokines and appears inducible and independent of calcium-calmodulin activation in vitro because calmodulin is tightly bound to the enzyme (1).

We tried to identify the localization and distribution of the constitutive isoform of e-NOS in the placenta of PIH and compare the expression of e-NOS in PIH with that in normotensive pregnancies.

MATERIALS AND METHODS

Tissue collection

The study groups consisted of 121 women admitted for preeclampsia to the Obstetrics Department of Severance Hospital during one year from January 1, 1995 to December 31, 1996. All had blood pressure values above 140/90 mmHg and proteinuria above 0.3 g/24 hr. The criteria of severe preeclampsia has been as follows: 110 mmHg or higher diastolic pressure, 2+ or more persistent proteinuria, presence of convulsion or HEELP syndrome (hemolysis, elevated liver enzymes, and low platelets) or fetal growth retardation. Two pregnancies were complicated by HEELP syndrome. Three pregnant women had diabetes mellitus. No patient has previous hypertension or history of drug abuse. Forteen placentas in preeclampsia were twin pregnancy. All babies except two less than 24 weeks gestational age were delivered by C-section. A comparison group composed of 14 normotensive pregnant women was also studied. We intended that both groups would be similar in age and gestational age. One of the women had idiopathic thrombocytopenic purpura (ITP) with severe anemia.

Immunohistochemical stain

Paraffin sections for immunohistochemical staining were mounted on slides coated with poly-L-lysine and stained with polyclonal antibody against endothelial NOS (Transduction Laboratory, Kentucky, U.S.A.). Briefly, slides were deparaffinized, rehydrated. They were immersed in 10 mM sodium citrate, pH 6.0, and heated two times in a microwave oven (700 watts) for five minutes each time and sequentially placed in running cold water with soaking PBS (pH 7.2) for 15 minutes. Subsequently, a circled marking was written by Dako pencil on all sections and blocked with 3% hydrogen peroxide for 10 minutes. Sections were sequentially incubated with normal goat serum for 30 minutes, a 1:350 dilution (1 μg/ml) of e-NOS primary antibody overnight at 4℃ respectively. Sections were additionally incubated at room temperature for 30 minutes, sequentially with biotinylated goat anti-mouse IgG for 30 minutes at room temperature, and with ABC reagent (Dako, Carpinteria, CA, U.S.A.). 3-amino-9-ethyl-carbazole (Sigma, St. Louis, MO) was used as chromogen with hematoxylin counterstain. Vessels of umbilical cord were all intrinsically used as a positive control.

Quantification of the immunoreaction for e-NOS

In the score proposed by McCarty et al., both the intensity and the distribution of staining area were incorporated in a single numerical score. The score, $\sum=(i+1)\times Pi$, consists of the sum of the staining intensities, i (0, 1+, 2+, 3+) multiplied by the percentaqge of cells, P (0, 1, 2, 3, 4) in each category of staining. The immunoreactive score (IRS) for semiquantitative estimation of the immunocytochemical reaction (18) is presented in Table 1.

Table 1. Immunoreactivity score (IRS) for semiquantitative estimation of the immunocytochemical reaction

IRS=SI×PP	
SI (staining intensity)	0=negative 1=weak 2=moderate 3=strong
PP (Percentage of positive cell)	0=negative 1=10% pos.cell 2=10-50% pos.cell 3=51-80% pos.cell 4=80% pos.cell
Maximum IRS: 3×4=12	

Statistical analysis

All statistical analysis was done using the Windows SPSS PC version 6.1. T-test was performed for comparisons of e-NOS expression between the control and PIH group. Correlation was investigated to compare between two continuous parameters such as e-NOS expression and gestational age or patient age. The interval variance of patient age and gestational age was grouped by 5 years and 5 weeks respectively. Multiple regression test was also done to determine which combinations of several kinds of variables were the most statistically significant independent factors to the expression of e-NOS.

RESULTS

The characteristics of all the women and their babies are summarized in Table 2. Major proportion of PIH patients suffered from severe PIH (81.8%). Patient age and gestational age between the preeclampsia and control group showed no significant differences. The localization of e-NOS in the cotyledons was identified by granular and punctuate cytoplasmic staining predominantly within the syncytiotrophoblasts surrounding the terminal villi (Fig. 1) and in a minor proportion of endothelial cells of

Table 2. Clinical information of studied group

Variables	PIH				Control	(%)
	Mild	(%)	Severe	(%)		
Number	22	(18.18)	99	(81.82)	14	
Age (yr : mean±S.D.)	28.32±4.65		30.37±4.12		30.57±5.55	
(minimum-maximun)	(22-39)		(20-42)		(20-40)	
Gestational age (weeks : mean±S.D)	37.41±2.23		34.57±4.00		32.29±6.59	
(minimum-maximun)	(33-41)		(19-41)		(15-40)	
Fetal distress (n)	3	(13.63)	23	(23.23)	0	
Intrauterine fetal death (n)	0		5	(5.05)	1	(7.14)
Intrauterine growth retardation (n)	1	(4.55)	8	(8.08)	0	
Abruptio placenta (n)	0		5	(5.05)	2	(14.29)
Placenta accreta (n)	0		6	(6.06)	1	(7.14)
Meconium staining (n)	2	(9.09)	8	(8.08)	0	

All values are mean ± standard deviation (S.D) or number of frequency (n).
Min : minimal value, Max : maximal value, yr : years, wk : weeks

vessels in stem villi or decidua. The distribution and intensity of e-NOS expression was remarkably prominent in the placenta of early gestational age, not in the normotensive term placenta. No immunostaining was identified in the capillaries within the terminal villi or trophoblast island comprising X-cell identical to cytotrophoblast. Only one case showing chorangiosis of 34 weeks gestational age from normotensive placenta revealed prominent expression of e-NOS in the capillaries of terminal villi (Fig. 2). The expression of e-NOS in an umbilical cord from normotensive placenta was identified, in general, in the endothelium and concentric media of the umbilical arteries and veins (Fig. 3). Whereas no difference in the localization or the intensity of the e-NOS immunostaining was seen in the endothelium of umbilical arteries from preeclampsic placentas compared with those from the normotensive placentas, the difference of e-NOS immunostaining in the syncytiotrophoblast facing the intervillous space was chiefly quantitative and partly qualitative between the two groups. The localization and intensity of e-NOS immunostaining in preeclampsia was significantly quite less and weaker than in control (Fig. 4A and B). Tanny-Parker knots in PIH were revealed to be very weak for

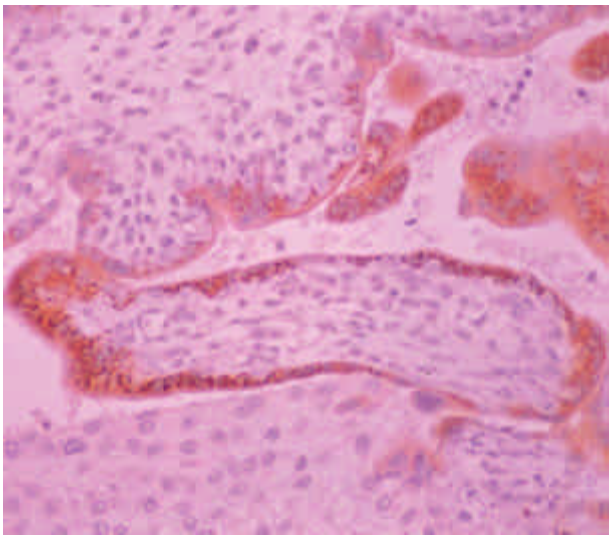

Fig. 1. Expression of e-NOS in normotensive placenta. Notice strong cytoplasmic signals with punctuate pattern predominantly in the syncytiotrophoblast facing the intervillous space (AEC, ×200). There is no expression of e-NOS in a single cell or capillary in the intravillous stroma.

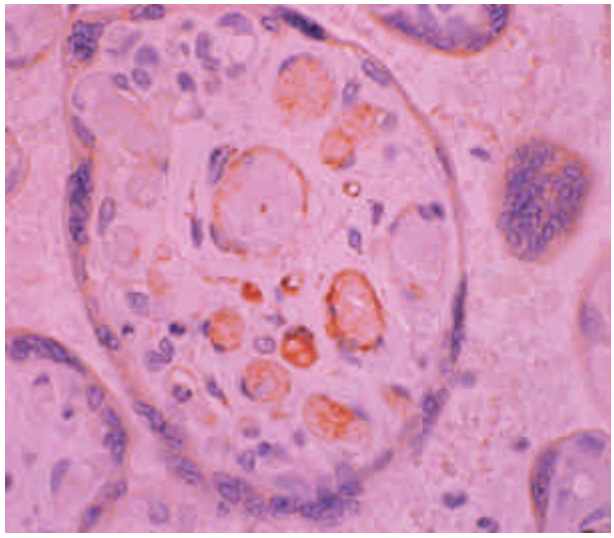

Fig. 2. Expression of e-NOS in chorangiosis. A normotensive placenta of 34 weeks of gestational age revealed chorangiosis. Numerous intravillous vessels lined by endothelial cells are stained for eNOS. Notice the relatively weak expression of e-NOS in other adjacent intravillous capillaries (AEC, ×200).

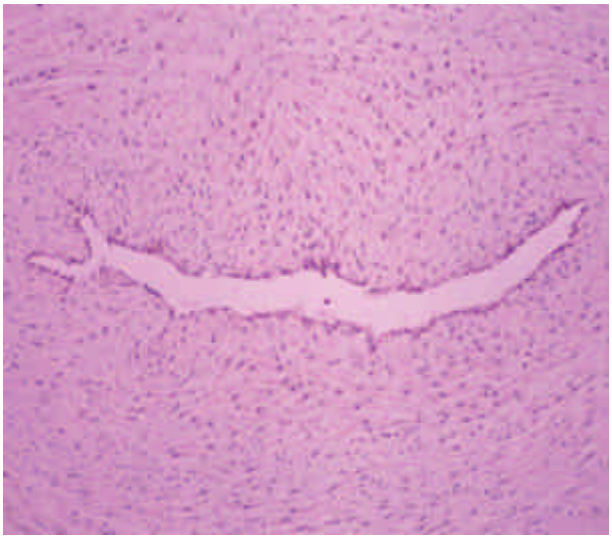

Fig. 3. Expression of e-NOS in an umbilical cord. Endothelial cells facing the lumen are definitely positive for e-NOS. The smooth muscle cells concentrically layered are also positive (AEC, ×200).

e-NOS in contrast to syncytial knots in control placenta (Fig. 4C). Decidual vasculopathy (Fig. 5A) characteristic of PIH, atherosis or medial fibrinoid deposition, never revealed e-NOS immunostaining (Fig. 5B) in comparison with weak minimal e-NOS expression in the spiral arterioles of decidualis parietalis or basalis. Even in normal pregnancy, one placenta showed definite evidence of decidual vasculopathy in decidua vera as well as decidua basalis, which was confirmed to be associated with longstanding idiopathic thrombocytopenic purpura and anemia, but no evidence of previous hypertension. The incidence of decidual vasculopathy showed an increased tendency with the severity of PIH. The e-NOS expression between two groups were significantly different, however, there was no difference within the PIH group (Table 3). In the multivariated analysis of 135 specimens from PIH and normal women for whom data were available on patient age, gestational age, intrauterine fetal death, abruptio placenta, placenta accreta, severity, fetal distress, meconium staining and intrauterine growth retardation, only the presence or absence of preeclampsia and status of abruptio, proved to be

Table 3. Comparison of pathologic findings bewteen the PIH and control placentas

Pathological findings	PIH				Control (n=14)	(%)
	Mild (n=22)	(%)	Severe (n=99)	(%)		
Decidual vasculopathy (No)	2	(9.09)	21	(21.2)	1	(7.14)
e-NOS expression	3.36±3.35		3.6±3.24		7.71±4.16*	

The value of e-NOS means the mean value (IRS) ± standard deviation.
* means that e-NOS expression is significantly higer than in PIH (p-value=0.000, by t-test with equal variance)

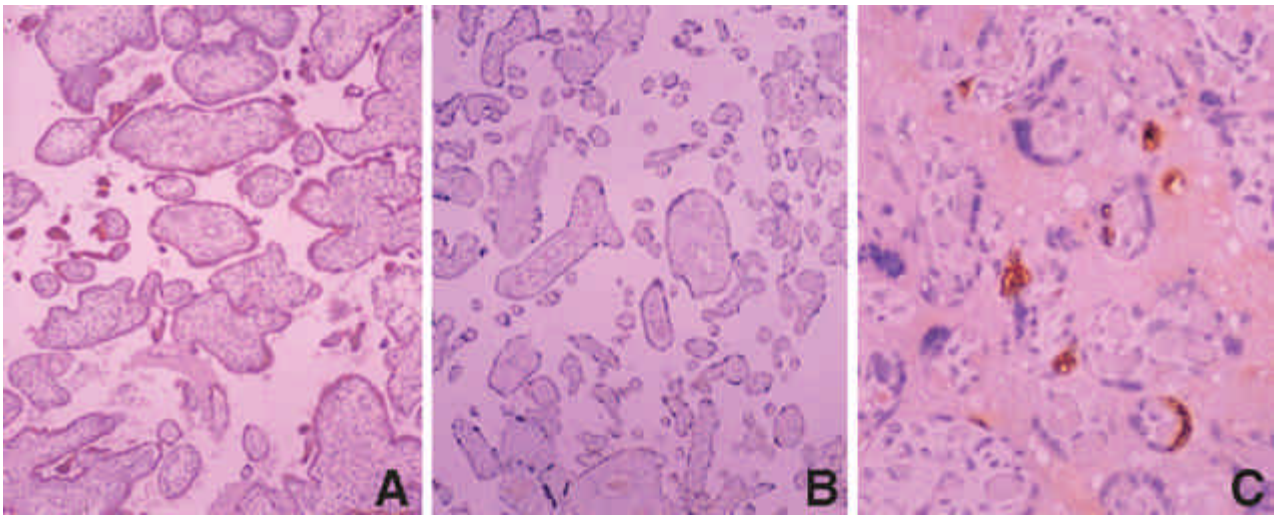

Fig. 4. Comparison of e-NOS in preeclampsia with normotensive placenta. **A :** E-NOS stained in the syncytiotrophoblast with strong intensity. **B :** Minimal expression of e-NOS in the hypermature palcenta of preeclampsia. **C :** Tanny-Parker knots showing focal crescent-like expression or absolutely negativity for e-NOS (A and B: AEC, ×100, C: AEC, ×200).

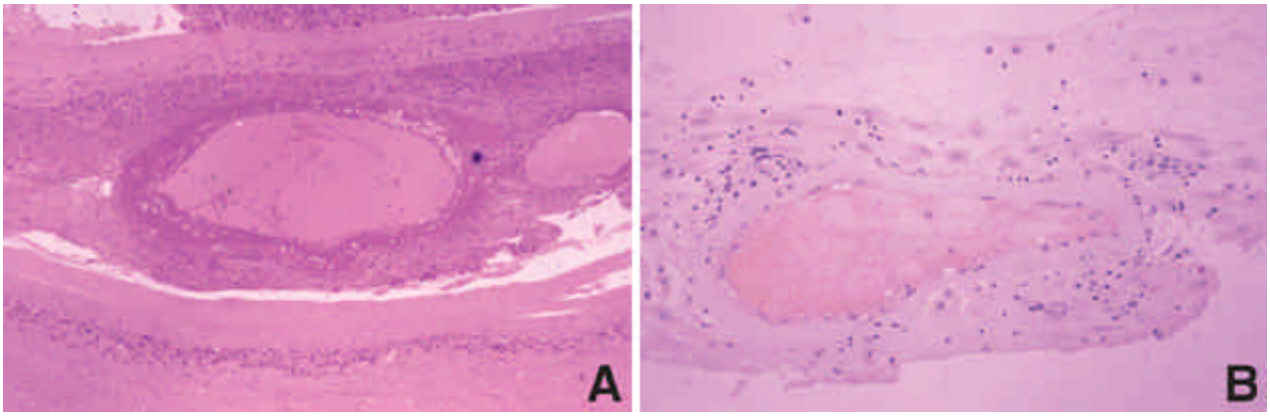

Fig. 5. Light microscopic findings of decidual vasculopathy in preeclampsia. **A :** Medial fibrin deposition and subintimal foam cell deposition in the vessels of decidual parietalis are demonstrated, which is one of the characteristic signs of preeclampsia. **B :** Absolutely negativity for e-NOS in the affected vessels (A: hematoxylin-eosin, ×200, B: AEC, ×200).

Table 4. e-NOS expression according to the status of variables

Variables	Presence	Absence	Beta	P-value
Age			.969094	.0598*
Gestational age			.930278	.7005
Preeclampsia	3.55 ± 3.27	7.71 ± 4.16	-.299867	.0005**
Abruptio	8.43 ± 4.27	3.74 ± 3.39	.223338	.0074**
IUFD	3.5 ± 4.27	4.00 ± 3.56	.988515	.7005
IUGR	3.73 ± 3.28	4.05 ± 3.67	.980694	.8687
Fetal distress	3.11 ± 3.00	4.05 ± 3.63	.952566	.3369
Accreta	2.43 ± 2.32	4.07 ± 3.63	.988295	.3576
Meconium staining	4.3 ± 4.36	3.96 ± 3.53	.980694	.7168

The values are correspondent to the mean ± standard deviation.
The beta and p-value were obtained from the linear multiple regression test by setting of independent factor on e-NOS value (spss v6.0).
** means significant factor.
* means more or less significant factor.

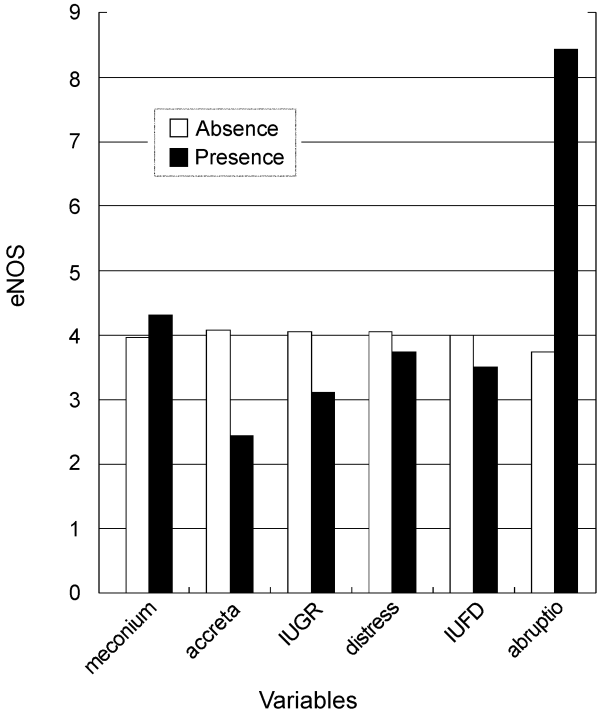

Fig. 6. Comparison of e-NOS expression according to the status of variables.

significantly correlated with the e-NOS expression (linear multiple regression test : spss 6.0, vs severity; b= -.278294, p=0.0009; vs abruptio; b=.223338, p=.0074 respectively). Even though not proven statistically, the expression of e-NOS showed a strong tendency to increase as the age of the patients increased (b=.969004, p=.0598). These results are summarized in Table 4 and Fig. 6.

DISCUSSION

NO radical, an inorganic free-radical gas, plays a major role in the maintenance of low vascular resistance and in attenuating the action of vasodilators in the fetal circulation of the placenta (1-15). NO relaxes vascular smooth muscle and inhibits aggregation and adhesion of platelets by raising intracellular cGMP (15). Moreover, there is a clear synergism between the anti-aggregatory effects of prostacyclin and subthreshold concentration of NO. Clearly NO is the local hormone, only effective on, or within the immediate vicinity of, the cell which release it. Any that escapes into the blood stream would decay chemically within a few seconds to form nitrite, were it not immediately inactivated by hemoglobin (17). This reason makes it difficult to qualify NO directly. Instead of NO measurement, plasma and urinary nitrite and nitrate, stable oxidative products of NO, have been

measured (19). In addition, through the use of NO synthase (NOS), NO synthesis also can be studied indirectly. NOS are the enzymes that catalyze the production of NO from L-arginine (1). Vascular endothelium, brain, and macrophages were the first mammalian systems in which NO synthesis was described (1, 16). The research on NOS has proceeded with remarkable speed, being first described in 1989, purified in 1990, and cloned in 1991 (17). The e-NOS has been called constitutive because the enzyme is constantly present in cells, awaiting activation by a variety of agonists such as acetylcholine (1, 11-17). The majority of e-NOS in pregnancy has been carried out in animal models. In human studies, e-NOS has been isolated from placental extracts (20), villous vasculature (21) and syncytiotrophoblast (22). From their study of isolated perfused human placenta, Myatt et al. (23) found that NO attenuates the action of vasoconstrictors, and they concluded that NO production could be stimulated by hemodynamic forces. In our study, we also demonstrated e-NOS localization chiefly along the syncytiotrophoblast layers, with umbilical arteries and veins, and not only in endothelial cells but also in the smooth muscle cells concentrically oriented in media. Attempts to extend this model of diminished NO synthesis to human preeclampsia have been limited, and furthermore the results were controversial. The indirect methods to reflect NO synthesis include measurement of urinary cGMP excretion or serum nitrate and nitrite level. However, these indirect methods have several limits for elucidating the nature of e-NOS because the majority of NO synthesis in the human body probably results from the activity of neuronal constitutive isoenzyme (1).

The method using immunohistochemical staining was documented by Ghabour et al. (16), and labelled by fluorescence. They documented that the major difference of e-NOS expression between PIH and normotensive placenta was that the e-NOS is stained diffusely in apical cytoplasm of syncytiotrphoblast in comparison with the intense, basal and more punctuate in nomal placenta. Moreover, e-NOS derepress in the endothelium of terminal capillaries of villous tree from PIH, but not in similar vessels from the normotensive counterparts. Our study also demonstrated that e-NOS expression is much more intense and punctuate in the control group than in PIH. In addition, we compared the degree of e-NOS expression more objectively than usual. The major difference of e-NOS expression lay on the focality and intensity, thus the immunoreactivity score for semiquantitative estimation, the value of staining intensity multiplied by percentage of positive cells, was significantly lower in the sections of cotyledons from preeclampsia than in those from the normotensive placentas. This difference showed a remarkable contrast in the Tanny-Parker knots in the case of PIH in comparison with the true syncytial knots from near term placenta of normotensive counterparts. Another expected result was that the vessels affected by atherosis or decidual vasculopathy, never expressed e-NOS in comparison with minimal expression of e-NOS in the endothelium of decidual vessels from the normotensive placentas.

We intended to detect which factors are correlated with the difference of e-NOS expression. Generally, the principal pathologic findings of preeclampsia are known for Tanny-Parker knots, decidual vasculopathy, with central infarct and abruptio placenta. In addition to these quite specific findings, several clinical parameters, including patient age, gestational age, fetal distress, accreta, severity, fetal death, intrauterine growth retardation and meconium staining were considered in our study design. The degree of e-NOS expression was proven to be correlated with few variables by statistical analysis. The meaningful variables documented by regression study included the severity, abruptio placenta and age. Descriptively speaking, the PIH placenta from younger patients who lack abruptio placenta showed meaningfully decreased expression of e-NOS in comparison with counterparts. Though the fact that PIH expresses lower e-NOS volume is predictable and explainable, the result of decreased e-NOS expression in younger age and the lack of abruptio can not be explained satisfactorily. But it seems that PIH presenting abruptio revealing higher e-NOS expression may be caused by compensation followed by severe disturbance of blood flow. Nontheless, gestational age was unexpectedly far from the range of statistical significance, though it is still unknown whether gestational age may have also contributed to this difference because the preeclamptic women were, on average, 5-6 weeks earlier in pregnancy than their normal counterparts (1). As matter of a fact, the normotensive placenta from earlier gestational age expressed far more and stronger immunoreaction of e-NOS than near-term placenta.

The fact that syncytiotrophoblast expresses low e-NOS results in platelet and leukocyte adhesion and consequently in the intervillous thrombi (16, 24). In addition, the fact that the expression of e-NOS is decreased semiquantitatively in case of preeclampsia appears to mean that the total mass of e-NOS is relatively low and resultantly enzymatic function could also be deprived. It may denote that the e-NOS decreases in cotyledons of preeclampsia and thus have something to do with the pathogenesis of preeclampsia. Neither umbilical cord arteries, veins, nor chorionic plate vessels showed any apparent difference in the localization or intensity of e-NOS immunostaining between the preeclamptic and normotensive placentas.

In conclusion, this study confirms that the degree of e-NOS expression is far lower in preeclampsia with the exception of abruptio of younger age than in normotensive placenta. We speculate that these differences could mean alteration of the local regulation of fetoplacental interface in preeclampsia.

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