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—Original Article—

Expression and Localization of NO Synthase Isoenzymes (iNOS and eNOS) in Development of the Rabbit Placenta

Hamayun KHAN[1)], Ken Takeshi KUSAKABE[1,2)], Shoichi WAKITANI[1)], Masato HIYAMA[1)], Ai TAKESHITA[3)] and Yasuo KISO[1,2)]

[1)] *Laboratory of Basic Veterinary Science, The United Graduate School of Veterinary Science, Yamaguchi University, Yamaguchi 753-8515, Japan*

[2)] *Laboratory of Veterinary Anatomy, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753-8515, Japan*

[3)] *Laboratory of Laboratory Animal Science, Division of Veterinary Science Animal Science, Graduate School of Life and Environmental Science, Osaka Prefecture University, Osaka 598-8531, Japan*

Abstract. Nitric oxide synthase (NOS) is a key regulator of angiogenesis and embryogenesis in the mammalian reproductive process. Here, we attempted to clarify the expression and localization of inducible and endothelial NOS (iNOS and eNOS) in the developing rabbit placenta. Real-time RT-PCR analysis indicated that iNOS mRNA was significantly upregulated till the complete development of the placenta (d18), and then significantly decreased at the end of fetal growth stage (d28) during successful pregnancy. The eNOS mRNA was also enhanced in the pregnant uteri and gradually decreased near the term of pregnancy. Western blot analysis also showed elevation of the iNOS and eNOS protein levels during the course of successful pregnancy till the functional maturation of the placenta (d18). Immunohistochemical study revealed distinct localizations of iNOS along the radial arteries and eNOS at the spiral arteries and arterial sinuses in the developing placenta. This may reflect that iNOS and eNOS participate in pregnancy success through placentation-specific vascular formation and by supporting adequate blood circulation in the rabbit placenta.

Key words: eNOS, iNOS, Rabbit placenta, Vascular formation

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In pregnant mammals, the placenta acts as exchange interface for nutrients and waste products between the fetal and maternal circulation. The placenta is a very fast growing tissue with corresponding high metabolic demand from the embryo or fetus that requires an active blood supply and rapid vascular development [1–3]. Failure of placental growth during early and mid pregnancy is directly associated with inadequate uterine and umbilical blood flow, which adversely affects transportation of fetal nutrients [4]. Extensive increase of the transplacental exchange during the last half of gestation is closely dependent upon the dramatic growth of the vascular architecture and the resultant large volume of uterine and umbilical blood flow [1].

Nitric oxide (NO), a multifunctional biomolecule, is produced from the essential amino acid L-arginine via nitric oxide synthase (NOS), which is classified into the calcium-independent or constitutive calcium/calmodulin-sensitive isoforms. The former is represented by inducible NOS (iNOS), and the latter is represented by the endothelial and neuronal NOS (eNOS and nNOS) [5]. NO plays crucial roles in the mediation of a wide variety of physiological processes including vasodilation, angiogenesis, platelet aggression, immune functions, connective tissue remodeling and smooth muscle activity [6]. iNOS and eNOS are known to dynamically regulate normal physiological events during successful pregnancy such as ovulation, implantation, trophoblast invasion, placental formation, fetal development and delivery [7–9].

In the developing placenta, specific vascular formation occurs through the processes of destruction of preexisting vessels, *de novo* angiogenesis and convergence of blood path in association with the invitation of plenty blood flow and efficiency of nutritional exchange. The NO produced by NOS serves as an enhancer of proliferation and migration in endothelial cells and additionally as an inhibitor of apoptosis and the anti-angiogenesis factor angiostatin [10, 11]. Recently, we observed unique and dynamic vascular formation of such things as the maternal arterial sinuses, maternal radial arteries and fetal labyrinthine capillaries of the developing rabbit placenta [12]. The arterial sinus has a thickened smooth muscle wall, showing a functional association of NO with the control of local blood flow. With the advancement of rabbit pregnancy, vascular remodeling in the maternal and fetal vessels becomes dominant, as shown by the narrowing intervillous spaces and drastic ramifications in the labyrinth. Recent studies in various mammalian species indicate that NO acts as a major modulator of uterine blood flow during pregnacy [13], placental-fetal blood flow [14] and placental angiogenesis [2].

The distribution of iNOS and eNOS has been confirmed in the placentas of many animal species including the human [15], rat [16], sheep [3, 17] and rhesus monkey [18]. In spite of the fact that the rabbit is a useful research model in the field of reproductive and developmental biology, previous published literature is absolutely silent about the assessment of iNOS and eNOS in the rabbit placenta.

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Correspondence: KT Kusakabe (e-mail: kusakabe@yamaguchi-u.ac.jp)

In the present study, we demonstrated a specific expression profile and vascular localization of iNOS and eNOS, indicating a certain reproductive role in the developing rabbit placenta.

Materials and Methods

Animals

A total of 20 female rabbits (Japanese White) bred under conventional conditions in the animal facility of Kyudo (Saga, Japan) were purchased; the animals were at days 8 (peri-implantation period), 13 (commencement of placental development), 18 (completion of placental development) and 28 of pregnancy (end of the fetal growth stage and near term) or nonpregnant at the time of purchase. The mating day was designated as day 0 of pregnancy. Rabbits were sacrificed by the intravenous injection of 130 mg/kg pentobarbital sodium. Nonpregnant uteri and placentas were immediately frozen at –80 C or fixed with Zamboni's solution (0.21% picric acid and 2% paraformaldehyde in 130 mM phosphate buffer). The experimental protocols and animal use were approved and followed in accordance with the Yamaguchi University Animal Experimental Guidelines.

Real-time RT-PCR

Total RNA was extracted from frozen tissues using Isogen RNA extraction reagent (Nippon Gene, Tokyo, Japan). From 1μg of total RNA, cDNA was synthesized with ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan). Appropriate primers and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) were used for the real-time PCR reaction with a StepOnePlus Real-Time PCR System (Applied Biosystems). Primers were designed using the Primer Express software (Applied Biosystems) based on the sequence of each rabbit target gene in a published database and the primer specificity was validated by BLAST (Table 1). Data were evaluated by the $\Delta\Delta C_T$ method of relative quantification using the StepOne Software (Applied Biosystems) following normalization by GAPDH as an internal control.

Western blot

Frozen samples were homogenized with the extraction buffer (125 mM Tris-HCL pH8.0, 2 mM $CaCl_2$; 1.4% triton X-100) and protease inhibitor cocktail (cOmplete, Mini, Roche Diagnostics, Indianapolis, IN, USA). Homogenates were kept on ice for 30 min and then centrifuged at 15,000 × *g* for 10 min at 4 C and the supernatants were collected. Following measurement of total protein concentrations using an RC DC protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA), samples were diluted with Laemmli sample buffer (2% SDS; 62.5 mM Tris-HCl, pH 6.8; 25% glycerol and 0.01% bromophenol blue) in equal amounts, and applied to polyacrylamide gel electrophoresis and semidry blotting on a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membranes were then treated with 5% nonfat dried milk overnight at 4 C and immunoblotted for 1 h with primary antibody. Because of limited availability of an appropriate commercial antibody and high homogeneity of the amino acid sequences between human and rabbit NOS (96% in iNOS and 99% in eNOS), mouse monoclonal anti-human iNOS and goat polyclonal anti-human eNOS antibody (both from R&D Systems, Minneapolis, MN, USA) were selected as the primary antibodies. Membranes were then incubated with biotin-conjugated anti-mouse or anti-goat IgG (Vector Labs., Burlingame, CA, USA). Following by incubation with avidin-biotin-complex reagent (Vector Labs), immunoreactive blots were identified using chemiluminescence detection with an ECL Plus kit (GE Healthcare, Buckinghamshire, UK) and LAS- 3000 luminescent image analyzer (Fujifilm, Tokyo, Japan). Cross-reactivity of primary antibody was confirmed by the molecular weight of the reactive bands. GAPDH was monitored as a standard reference, using a mouse monoclonal anti-GAPDH antibody (Chemicon, Temecula, CA, USA). The band intensity was analyzed using the NIH Image analysis software (NIH, Bethesda, M D, USA).

Immunohistochemistry

Paraffin sections prepared from fixed samples were used for immunohistochemical analysis. Briefly, deparaffinized sections were rehydrated and subjected to antigen retrieval by using 10 mM citrate buffer (pH 6.0) and a microwave oven. After quenching of endogenous peroxidase and blocking with non–fat dry milk, the primary antibody (goat anti-human eNOS or mouse monoclonal anti-human iNOS, both from R&D Systems) was applied to sections overnight at 4 C. After several washes, the sections were incubated with biotin-conjugated anti-goat or anti-mouse IgG antibody (Vector Labs). A Vectastain Elite ABC kit (Vector Labs) was used to detect the secondary antibodies, and then diaminobenzidine (DAB) was applied for visualization. Sections were briefly counterstained with Mayer's hematoxylin.

Statistical analysis

Results are reported as means ± standard deviation (SD). The relative expressions of mRNA and protein levels were evaluated by one-way ANOVA using SPSS 16.0 for windows statistical package (SPSS, Chicago, IL, USA). When the difference was significant ($P < 0.05$), comparisons between groups were analyzed using the Tukey-Kramer post hoc test.

Results

Expression of iNOS and eNOS in rabbit placentas

Quantitative PCR analysis indicated that both NOS isoenzymes showed a pregnancy-dependent increase in the rabbit placenta (Fig. 1). The iNOS mRNA expression showed a significant increase in placentas at d8 (peri-implantation period) in comparison with nonpregnant uteri. The iNOS mRNA level was gradually elevated until d18 (completion of placental development), whereas a significant decrease was observed at d28 (the end of the fetal growth stage). The eNOS expression was also significantly increased in d8 placentas compared with nonpregnant uteri, and the mRNA level was maintained during the entire period of pregnancy until d28.

Western blot experiments demonstrated the presence of protein of the NOS isoforms at the rabbit placentas (Fig. 2). Although the amino acid sequence has not been fully confirmed, the molecular size of the rabbit iNOS has been demonstrated as 130 kDa [19]. On the other hand, molecular size of the rabbit eNOS has been calculated as 134 kDa based on information in a public protein

Table 1. The sequences (5' to 3') of the primers used in the real-time RT-PCR

Name	Forward	Reverse
iNOS	TGACGTCCAGCGCTACAATATC	GCGTCTCCAGTCCCATCCT
eNOS	GCCTCACTCATGGGCACAGT	TCGGAGCCGTACAGGATTG
GAPDH	TCACCATCTTCCAGGAGCGA	CACAATGCCGAAGTGGTCGT

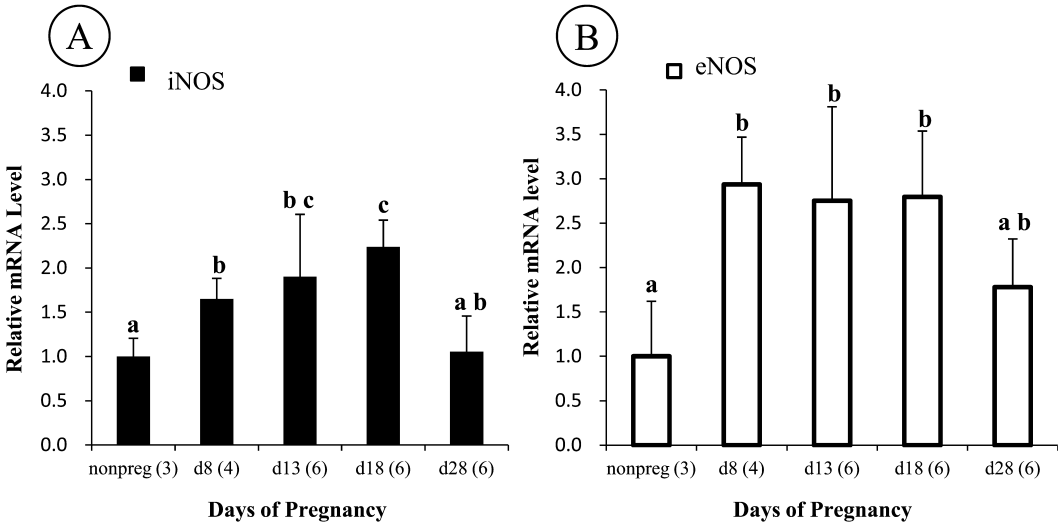

Fig. 1. Expression of iNOS (A) and eNOS (B) in the rabbit nonpregnant uteri and placentas. Real-time RT-PCR analysis was quantitatively evaluated. Different Letters indicate significant differences (a–c). Each bar represents the mean and SD. The number of specimens used is shown in parentheses. nonp = nonpregnant uteri.

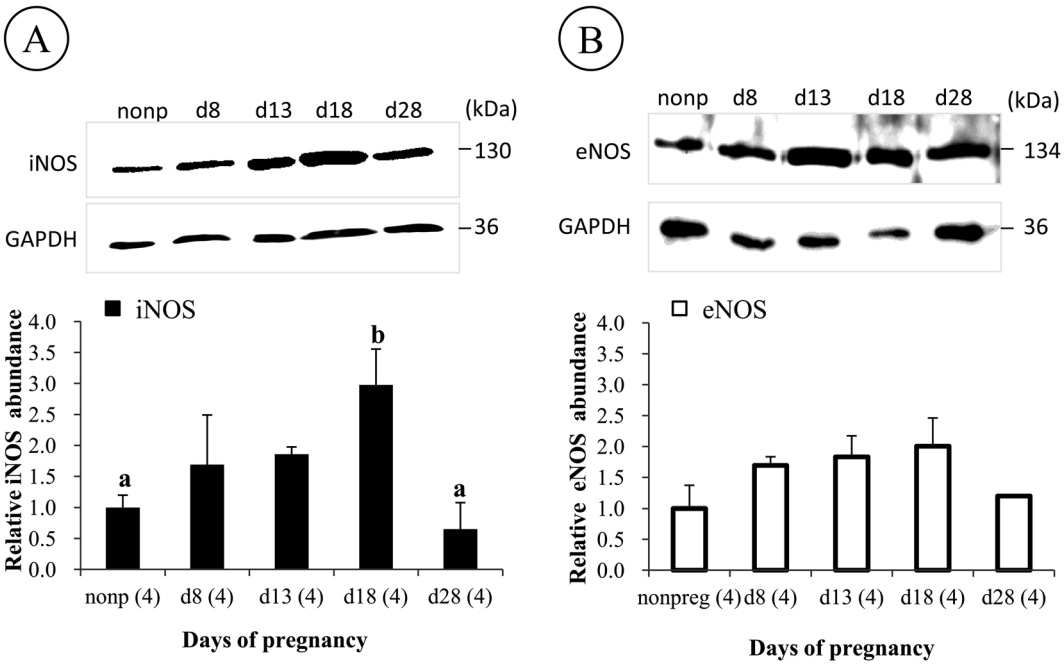

Fig. 2. Western blot analysis of iNOS (A) and eNOS (B) in the rabbit placenta. Representative data and a quantitative comparison are shown in upper and lower part of the figures, respectively. Each bar represents the mean and SD. Different Letters indicate significant differences (a, b). The number of specimens used is shown in parentheses. nonp = nonpregnant uteri.

database (UniProt, accession No. D7RVB4). In our experiments, both isoforms were detected with the expected sizes (upper column of Fig. 2). Placental iNOS increased during the early pregnancy period, showing significant elevation at d18 compared with non-pregnant uteri (Fig. 2A). In contrast, a significant decrease in iNOS protein was observed from d18 to d28. The protein level of eNOS showed a tendency to increased during early to mid pregnancy and decreased from d18 to d28 (Fig. 2B).

Localization of iNOS and eNOS in the rabbit placenta

An immunohistochemical study was carried out at two time points corresponding to the morphogenesis (d13) and structural completion

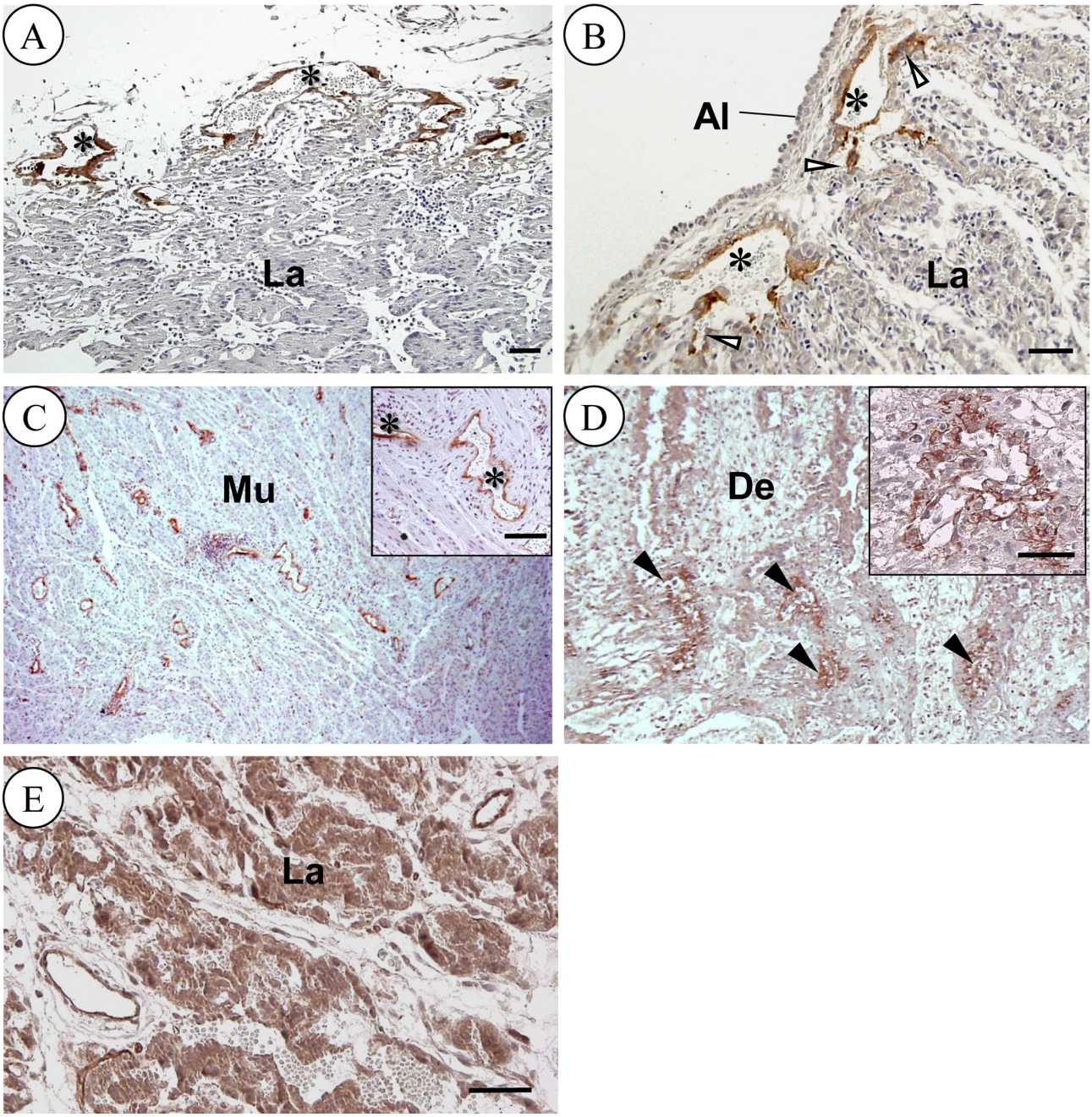

Fig. 3. Immunohistochemical studies of iNOS (A, B) and eNOS (C, D, E) in the rabbit placenta. A positive reaction was found for iNOS is found at d13 (A) and d18 (B) in the radial arteries (asterisks) located near the allantois (Al) and supplying maternal blood to the labyrinthine region (La). Their secondary branches shown at d18 are clearly positive for iNOS immunostaining (B, arrowheads). A positive reaction was seen for eNOS around the spiral arteries at d13 (C, asterisk) running through the uterine muscular layer (Mu). At d18, the eNOS-positive reaction extends to the arterial sinus (D, arrowheads) located in the basal region of the decidua (De). E) The trophoblast layer in the labyrinth region (La) showed a moderate positive reaction for eNOS at day 18. Bar scale=50 µm.

(d18) during placentation period (Fig. 3). The distribution of iNOS and eNOS was evident in different regions of maternal vessels of the rabbit placenta. A distinct immunopositive reaction of iNOS was observed in the endothelium of the radial artery trunks and their developing branches (Fig. 3A and B). These radial arteries send maternal blood to the intervillous spaces. An eNOS-positive reaction was detected in the endothelium of the spiral arteries at d13 (Fig. 3C) transversing through muscular layer and sending maternal blood to the arterial sinuses. At d18, an eNOS-positive reaction was detected around the vessels of the arterial sinuses (Fig. 3D), with reactivity being along the smooth muscular cells. Trophoblasts in the labyrinth region also showed moderate reactivity for eNOS at d18 (Fig. 3E).

Discussion

This is the first study to investigate the ontogenic changes of iNOS and eNOS in the developing rabbit placenta. Our findings in the current study indicated upregulation of both NOS isoenzymes during the placentation period and downregulation at the end of fetal growth. These data are similar to those reported in the placentas of rats, sheep and humans. In the rat placenta, iNOS mRNA has been demonstrated to increase significantly from d13.5 to d15.5 and decrease at d19.5, whereas the eNOS mRNA level does not show remarkable change [16]. The eNOS expression in the sheep placenta has been demonstrated to elevate during early pregnancy [20]. The expression of a high amount of iNOS mRNA has been reported in the human decidua and chorionic villi in the first trimester of pregnancy, suggesting that iNOS plays an important role for successful placentation and embryo development during early pregnancy [21, 22].

In the human placenta, NOS isoenzymes are also found in the placental vessels. The iNOS is distributed in arteriolar smooth muscle and the venous endothelium of umbilical vessels, and eNOS is present in the endothelial cells of umbilical and chorionic vessels [23–26]. In rats, a major expression site of iNOS is the interstitial and endovascular trophoblasts in the mesometrial triangle, and eNOS is found in the endothelium of fetal vessels of the placenta [27]. These reports indicate that NOS enzymes can be produced from smooth muscle or epithelial cells in the developing placental vessels, probably in association with vascular dilation or angiogenesis.

Rabbit placental vasculogenesis shows a distinctive structure in the arterial sinuses [12]. Arterial sinuses can be seen in the junctional region of placenta at around d13 and continue to expand until d18. It seems that arterial sinuses have the potential to retain maternal blood and supply it to fetal area constantly. The eNOS positive reaction was observed coincidentally with the maturation of the muscular layer around the arterial sinus. In our preliminary study, peripheral nerves for the regulation of muscular contraction in the arterial sinus could not be clearly demonstrated. The eNOS may regulate vascular tone of the arterial sinuses and send an appropriate volume of maternal blood. Furthermore, based on the rabbit placental formation, other regenerative maternal vessels also showed distribution of NOS isoenzymes. Both spiral and radial arteries are capital vessels that supply maternal blood to the arterial sinus and intervillous space. They showed endothelial reactivity for NOS isoenzymes, and regenerative branches of the radial arteries were clearly positive for iNOS in the endothelial layer. A muscular layer was confirmed around the spiral arteries and the trunks of radial arteries, but was absent at the branches of radial arteries. Thus, the distribution of NOS isoenzymes in the muscular layer or endothelium structure of these vessels may be associated with induction of vascular expansion or epithelial angiogenesis in the rabbit placenta.

Enhancement of blood flow is a primary mechanism for efficient transplacental exchange in normal pregnancy [28, 29] and is promoted by NOS in the ovine placenta [30]. Human intrauterine growth restriction (IUGR) during the third trimester is characterized by impaired uterine-placental-fetal blood flows, leading to reduced fetal nutrient uptake [31]. It seems conceivable that any alteration in expression of NOS isoenzymes can cause disturbance of the placental blood flow, resulting in IUGR as reported in humans [31]. The physiological significance and essentiality of placental NOS in the uteroplacental fetal system of various animal species has been demonstrated using NOS inhibitors, which resulted in the deficient angiogenesis of the developing fetus and placenta [32]. L-nitro arginine methyl ester (L-NAME) and asymmetric dimethylarginine (ADMA) are selective inhibitors of eNOS and iNOS respectively. In rats and guinea pigs, theses inhibitors result in serious variation in fetal development and preeclampsia-like biological responses, such as hypertension, proteinuria and fetal growth retardations [25, 33, 34]. Furthermore, our finding that placental NOS isoenzymes reduce drastically near term may reflect delivery initiation, which resembles results in the rat and mouse [16, 23, 35]. It has been proposed that NOS has a paracrine effect on discontinuance of myometrial flaccidity and preparation for partial contractility. NOS inhibitors would elucidate the precise role of NOS in the vascular systems in the rabbit placenta.

The expression of NOS isoenzymes can be regulated by cytokines such as interleukin (IL)-1 [36] or possible other cytokines abundant at the maternal-fetal interface including IL-4, IL-10, interferon (IFN) and leukemia inhibitory factor (LIF) [37]. IL-1 is particularly important as a mediator of inflammatory vascular dilatation induced by iNOS and eNOS [36]. In rats, IL-1β upregulates iNOS expression in trophoblast cells [38]. IFN-γ and LIF also induce iNOS in trophoblast cells and syncytiotrophoblasts in the human placenta [39]. Likewise, iNOS expression in human endometrial stromal cells can be enhanced by IL-1β and IFN-γ [40]. These cytokines, including IFN-γ and IFN-ω in rabbits [41], are considered to be engaged in the implantation-recognition process by advancing embryo infiltration and tissue remodeling of the endometrium via effects of NO and matrix metalloproteinase [18]. It seemed that induction of NOS isoenzymes is an underlying event in the developing placenta in the postimplantation period for initiation and reconstruction of placental vascular systems. Based on these reports, cytokines seem to have a possible role in the regulation of NOS expression in the developing rabbit placenta.

In conclusion, this study demonstrates differential expression of iNOS and eNOS in the rabbit placenta during the early to mid pregnancy periods. Localization of these nitric oxide synthases in different blood vessels of the developing placenta signifies divisional

cooperation in the process of vasculature regeneration and increase of maternal blood flow, which result in functional maturation of the rabbit placenta and healthy growth of the developing fetus.

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