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EXPRESSIONS AND REGULATION OF ENDOTHELIAL AND INDUCIBLE NITRIC OXIDE SYNTHASES IN MOUSE UTERUS DURING THE ESTROUS CYCLE AND EARLY PREGNANCY

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1. ABSTRACT

Nitric oxide (NO) has been implicated in many cellular processes. We examined the temporal and spatial expressions of endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) in mouse uteri during the estrous cycle and early pregnancy, as well as the regulation of eNOS and iNOS by estradiol (E_2) and progesterone (P_4) in ovariectomized mouse uteri using *in situ* hybridization and immunohistochemistry. Our results showed that positive eNOS and iNOS signals were localized in the uterine luminal epithelium and glandular epithelium during the estrous cycle. In ovariectomized mice, both E_2 and P_4 regulated the expression of eNOS and iNOS. During early pregnancy, eNOS and iNOS were detected not only in epithelium, but also in the primary decidual zone surrounding implanting embryos on day 6 of pregnancy, and in the whole decidualized stroma on day 7 of pregnancy. In conclusion, the results demonstrated that two NOS isoforms were localized in mouse uteri in specific temporal and spatial patterns during the estrous cycle and early pregnancy, and ovarian hormones can regulate their expression. Furthermore, the data suggest that the expression of NOS during the peri-implantation period might lead to enhance NO production, which could promote embryo implantation.

2. INTRODUCTION

Nitric oxide (NO), a major messenger molecule associated with blood vessel dilatation, immune function, and neurotransmission (1), is synthesized by nitric oxide synthase (NOS). To date, there are three distinct isoforms of NOS: neural NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). Among them, eNOS and nNOS, also called constitutive isoforms (cNOS), require calcium for activity, and are expressed constitutively under physiological conditions. The third isoform, iNOS, is not dependent on calcium for its activity, and is produced only in response to a variety of stimuli that include cytokines and immune stimulating factors (2).

Mammalian endometrium expresses numerous genes to maintain a regular estrous cycle and achieve a uterine environment that is optimal for embryo implantation. Multiple gene activation, driven by sex steroids, appears to be the major event responsible for the differentiation of endometrial cells, and evidence indicates that the gene regulation network within the endometrium plays a critical role in endometrial cell function during implantation and the estrous cycle (3). It has been suggested that NOS is expressed in mammalian reproductive tissues including the ovary, uterus, testis, and

epididymis, and that NO participates in a variety of reproductive processes, such as follicular development, ovulation, and spermatogenesis (4-6). The dysfunction in cyclicity in female eNOS-knockout mice further support the concept that eNOS/NO may have functional roles in ovulation and the estrous cycle (7). NOS levels were modulated by estradiol in the uteri of pregnant rats (8), but little is known about the expression patterns of eNOS and iNOS, and their regulation by sex steroids, in mouse uterus. Recent reports point to a role for the NO/NOS system in implantation, and studies have demonstrated that mouse uteri produce high levels of NO during the peri-implantation period (9). Our previous results showed that administration of a NOS inhibitor, N-nitro-L-arginine methyl ester (L-NAME), significantly reduced the number of implanted embryos in pregnant mice (10). In this study, we not only investigated the spatial and temporal expression patterns of eNOS and iNOS in the endometrial compartments of mouse uteri during the estrous cycle and early pregnancy, but also studied their regulation by ovarian hormones.

3. MATERIAL AND METHODS

3.1. Animals and tissue preparation

Investigations were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals for the Study of Reproduction filed by the State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences.

Adult mice of the outbred Kunming White strain (12-week age, 25-30 g weight) were provided by the Institute of Genetics, Chinese Academy of Sciences. Animals were bred at room temperature (RT, about 25°C) with controlled light cycles (12 hours of light and 12 hours of dark) and allowed free access to food and water.

The stages of the estrous cycle were identified by vaginal smear. The uterine horns were removed at different stages of the estrous cycle for *in situ* hybridization and immunohistochemistry (n=3 per stage).

To examine the effects of ovarian steroid hormones on eNOS and iNOS expression in non-pregnant mice uteri, mice were ovariectomized without regard to the stages of the estrous cycle. The mice rested for 2 weeks before receiving any treatment. The ovariectomized mice were divided into four groups and respectively injected with sesame oil (0.1 ml/mouse), E_2 (100 ng/mouse), P_4 (1 mg/mouse), and E_2 (100 ng/mouse) plus P_4 (1 mg/mouse) (11). Steroids were dissolved in sesame oil and injected s.c. with identical volumes. The mice were killed 24 h after injection. Uterine horns were collected for *in situ* hybridization and immunohistochemistry (n=3 per group).

Virgin female mice were allowed to mate with male mice. The morning of the day a vaginal sperm plug was found was designated day 1 of pregnancy. Pregnant mice were killed at 0900 h on days 1-7 of pregnancy, and embryos were recovered from the oviducts and uteri on days 2-4 to confirm pregnancy. Implantation sites were visualized following a tail vein injection of Trypan Blue dye solution [0.1% in saline (w:v)] on the afternoon of day 5. Uterine horns of the mice on days 1-7 of pregnancy (n=3 per stage) were excised for *in situ* hybridization and immunohistochemistry.

3.2. iNOS cDNA cloning

Total RNA was extracted from mouse uteri with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Oligonucleotide primers with an expected product of 682 bp were designed and synthesized based on the published cDNA sequence of mouse iNOS (GenBank accession no. M87039). The primer pair was 5′-GCTCGGGTTGAAGTGGTAT-3′ (sense) encompassing nt 1260-1278, and 5′-CTTGGTGTTGAAGGCGTA-3′ (antisense) corresponding to nt 1924-1941 of the mouse iNOS sequence.

The cDNA was synthesized using Superscript reverse transcriptase (Invitrogen, Carlsbad, CA) and oligo dT-primer from 2 μg of total RNA. The reaction was carried out at 42°C for 50 min and 70°C for 15 min. Amplification of the iNOS gene was performed in a 25 μl mixture containing 2 μl of reverse transcription products, 2 mM of $MgCl_2$, 200 μM of dNTPs, 10 pM of each iNOS primer, and 1 IU of Taq DNA polymerase (Invitrogen, Carlsbad, CA). The PCR was run for 30 cycles (denaturing at 95°C for 45 s, annealing at 56°C for 45 s, and elongating at 72°C for 45 s) followed by an extra 7 min at 72°C. The PCR products were separated on a 1% agarose (w:v) gel containing ethidium bromide. The amplified band was excised from the gel and purified using the CONCERT™ Rapid Gel Extraction System (Invitrogen, Carlsbad, CA). The purified fragment was then inserted into the pGEM®-T Easy vector (Promega Corp., Madison, WI). Some white colonies were identified by PCR using the above iNOS primers, and the inserted nucleotide sequences were sequenced from three different clones to exclude mutations generated by Taq polymerase. The sequencing was performed commercially (Songon Corp., Shanghai, China).

3.3. Hybridization probes

The eNOS plasmid was graciously provided by Dr. Philip A. Marsden (Department of Medical Biophysics, University of Toronto). To generate an antisense cRNA probe, the plasmid was linearized with Xba□and transcribed *in vitro* with T7 polymerase (Promega, Madison, WI). The sense probe was synthesized using Kpn I and T3 polymerase (Promega, Madison, WI). The iNOS plasmid was linearized with Pst I and transcribed *in vitro* with sp6 polymerase (Promega, Madison, WI) to produce the antisense probe, and the sense probe was synthesized using Nco I and T7 polymerase (Promega, Madison, WI). The cRNA probes were labeled with digoxigenin (DIG) RNA labeling mix (Roche Molecular Biochemicals, Mannheim, Germany). In brief, the labeling process was carried out at 37°C for 2 h followed by the addition of DNaseI to remove template DNA. The riboprobes were precipitated with LiCl and ethanol. The probe labeling efficiency was confirmed by a dot assay, and the purified

probes were stored in diethylpyrocarbonate (DEPC)-treated water at -80C.

3.4. *In situ* Hybridization

In situ hybridization was performed according to the method of Braissant and Wahli, with slight modifications (12). In brief, frozen sections (10 μm) on poly-L-lysine-coated slides were fixed for 15 min in freshly prepared 4% paraformaldehyde (PFA) (w:v) in phosphate buffered saline (PBS, pH 7.2), followed by two washes in PBS containing 0.1% active DEPC (v:v) for 15 min each at RT. After rinsing with 5 × SSC (1 × SSC is 0.015 M sodium citrate and 0.15 M NaCl) for 15 min, the sections were prehybridized with hybridization solution without probe [50% deionized formamide(v:v), 5 × SSC, and 120 μg/ml salmon sperm DNA] for 2 h at 56°C. The labeled probes were added to the prehybridization solution and denatured at 70°C for 10 min. 100 μl of the hybridization mix was pipetted onto each slide. Subsequently, the slides were placed into a moist chamber and hybridized at 56°C for 18 h. The sections were then incubated for 30 min in 2 × SSC at RT, then washed in 2 × SSC for 1 h at 65°C, then washed in 0.1 × SSC for 1 h at 65°C. The slides were incubated with anti-DIG-alkaline phosphatase (diluted 1:3000; Roche Molecular Biochemicals, Mannheim, Germany) for 2 h at RT and washed twice in buffer 1 (100 mM Tris, and 150 mM NaCl, pH 7.5) for 15 min at RT. Color development was carried out using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT-BCIP) reagents (Roche Molecular Biochemicals, Mannheim, Germany). To evaluate the background staining, DIG-labeled sense riboprobes were used for *in situ* hybridization under conditions identical to those used for the antisense probes. Results were recorded with the SPOT digital camera system (Diagnostic Instruments Inc., USA), and digital images were processed using Adobe PhotoShop (Version 5.5; Adobe, San Jose, CA).

3.5. Immunohistochemistry

Polyclonal antibodies against mouse eNOS and iNOS were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Immunohistochemistry was performed using a Zymed Histomouse-SP kit (Zymed Laboratories, San Francisco, CA). Briefly, cryosections (10 μm) were fixed for 10 min in 4% PFA (w:v) in PBS and washed. The sections were serially incubated with normal blocking serum for 10 min at RT, primary antibodies in PBS for 1 h at 37°C, and biotinylated secondary antibodies for 15 min at RT. The slides were treated with hydrogen peroxide [0.3% (v:v) in methanol] for 30 min to eliminate endogenous peroxidase activity, then incubated with HRP-Streptavidin for 15 min at 37°C. Intervening PBS washes were administered between incubations. The signal reaction was performed with a diaminobenzidine (DAB) kit (Zhongshan Corp., Beijing, China). Slides were washed, mounted, and examined by light microscopy. Slides incubated without primary antibodies were utilized as negative controls.

3.6. Image analysis

The intensity of *in situ* hybridization signals of iNOS and eNOS in uteri was quantified by the computer-aided laser scanning densitometry (Personal Densitometer SI; Molecular Dynamics, Sunnyvale, CA) and using the method described by Liu (13). In order to make the quantitative analysis credible in each sample, above 12 spots were randomly selected in luminal epithelium, glandular epithelium and stroma (decidua) for the sections hybridized to the antisense and sense probes. Specific signal intensity was defined as the average intensity for a section hybridized to the antisense probe minus the average intensity to the sense probe. Hybridization intensity was measured as the gray level within a given marked area that was above a preset gray threshold level. The same method was used to quantify the mmunohistochemical signals of iNOS and eNOS.

3.7. Statistical analysis

Statistical comparisons among groups were analyzed by one-way ANOVA followed by LSD test using SPSS software package (version 10.0.1, SPSS Inc., Chicago, IL, USA). Differences were considered significant at $P<0.05$. All values are presented as mean ± SEM.

4. RESULTS

4.1. iNOS cDNA cloning

The amplified 682 nucleotide fragments were sequenced to confirm their identities (GenBank accession no. M87039), and were found to share 100% sequence identity with the corresponding region of the mouse iNOS gene (nt 1260-1941).

4.2. Expressions of eNOS and iNOS in mouse uterus during the estrous cycle

In situ hybridization was performed to obtain a detailed picture of the spatial and temporal expression of eNOS and iNOS mRNAs in mouse uteri during the estrous cycle. The results was shown in Figure 1a-d and Figure 2. Figure 1a-d shows the restricted expression pattern of eNOS mRNA in the luminal epithelium, glandular epithelium, and stroma of non-pregnant mice during the estrous cycle. The signals for eNOS were strong during proestrous and estrous, but only moderate during diestrous and metestrous (Figure 2a, $P<0.05$).

As demonstrated by Figure 1e-h, the luminal and glandular epithelium of diestrous and estrous uteri showed strong hybridization signals for iNOS mRNA, while the level of iNOS mRNA was less intense during proestrous and metestrous (Figure 2b, $P<0.05$).

Immunohistochemical analyses were performed to examine the expression of eNOS and iNOS proteins in mouse uteri during the estrous cycle, and results showed that the localization of eNOS and iNOS proteins corresponded to that of their mRNAs during the estrous cycle. As shown in Figure 1i-l and Figure 2c, strong eNOS immunostaining was visualized in the luminal epithelium and glandular epithelium during proestrous and estrous, with diminished staining during diestrous and metestrous (Figure 2c, $P<0.05$). Immunoreactive iNOS expression was strong in the luminal epithelium and glandular epithelium

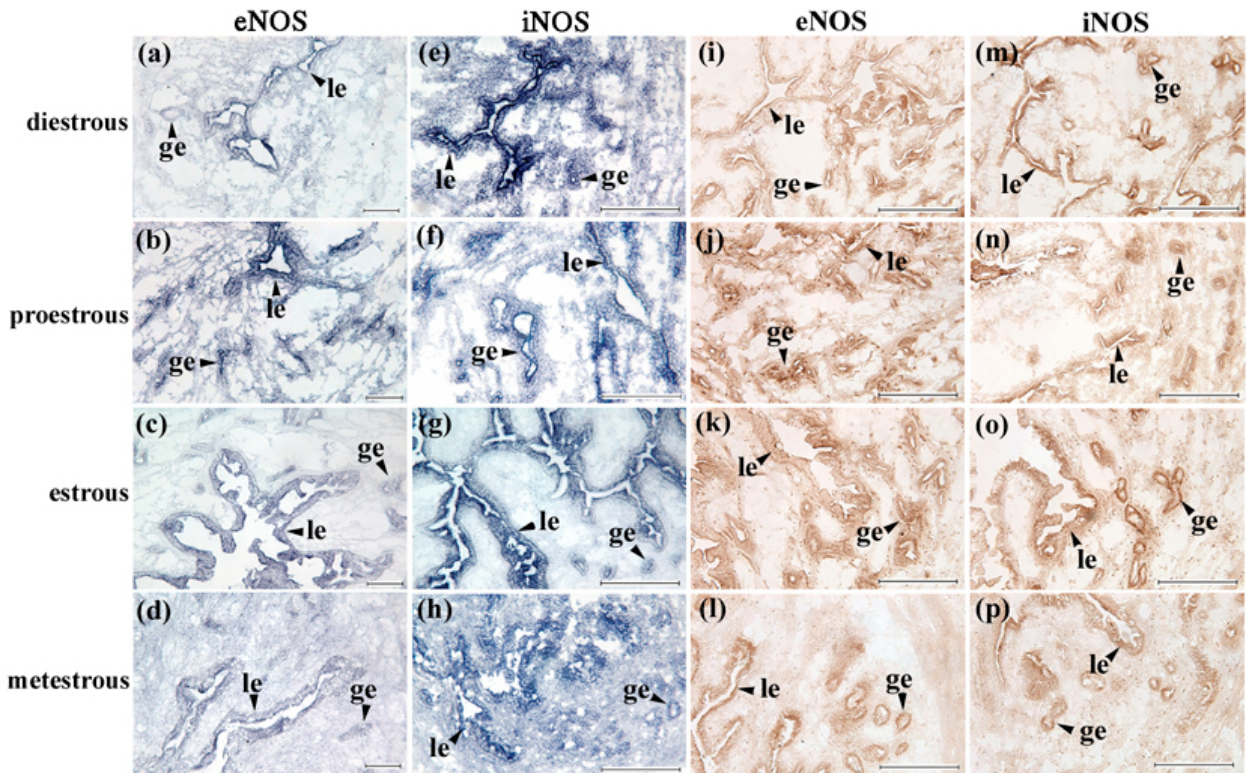

Figure 1. *In situ* hybridization and immunohistochemical detection of eNOS and iNOS expressions in mouse uteri during the estrous cycle. (a-d) *in situ* localization of eNOS mRNA during the estrous cycle; (e-h) *in situ* localization of iNOS mRNA during the estrous cycle; (i-l) immunohistochemical localization of eNOS protein during the estrous cycle; (m-p) immunohistochemical localization of iNOS protein during the estrous cycle; le, luminal epithelium; ge, glandular epithelium; bar=200 μm.

during diestrous and estrous (Figure 1m and o, Figure 2d, $P<0.05$), while only weak signals were detected in the luminal epithelium and glandular epithelium during proestrous and metestrous (Figure 1n and p, Figure 2d, $P<0.05$).

4.3. Effects of ovarian steroid hormones on uterine eNOS and iNOS expression in ovariectomized mice

The effects of ovarian steroid hormones on uterine eNOS and iNOS expression in ovariectomized mice are summarized in Figure 3 and 4. Ovariectomized mice treated with sesame oil served as control specimens, and signals for eNOS mRNA were barely discernible in the uteri of oil-treated mice (Figure 3a), while the levels of eNOS mRNA were significantly increased in the luminal and glandular epithelium of mice following the injection of E_2 (Figure 3b). The signals for eNOS mRNA in the luminal epithelium, glandular epithelium, and stromal bed were similarly elevated following P_4 treatment (Figure 3c). P_4 plus E_2 significantly increased the levels of eNOS mRNA in the luminal epithelium, glandular epithelium, and stromal bed (Figure 3d, and Figure 4a, $P<0.05$).

When compared with signals in the oil-treated ovariectomized mouse uteri, E_2 significantly stimulated iNOS mRNA expression (Figure 3e and f). The expression of iNOS mRNA was suppressed in the luminal epithelium, but was elevated in stroma, following P_4 treatment (Figure 3g). Coadministration of E_2 and P_4 profoundly increased the generation of iNOS mRNA in the glandular epithelium, luminal epithelium, and stromal bed (Figure 3h, and Figure 4b, $P<0.05$).

The signal for eNOS proteins was very weak in ovariectomized mice injected with oil alone (Figure 3i), but injection of E_2 resulted in a significant increase in eNOS protein content in the luminal and glandular epithelium (Figure 3j). Compared with the control, the eNOS protein signal in the glandular epithelium and stroma was strong following P_4 treatment (Figure 3k), and treatment with E_2 and P_4 together significantly increased expression of eNOS protein in the luminal epithelium, glandular epithelium, and stroma (Figure 3l, and Figure 4c, $P<0.05$).

In ovariectomized mice treated with oil alone, signals for the iNOS protein were weak (Figure 3m). E_2 administration resulted in a significant increase in iNOS protein content in the luminal epithelium and glandular epithelium (Figure 3n). Treatment with P_4 or coadministration of P_4 and E_2 stimulated iNOS protein generation in the luminal epithelium, glandular epithelium, and stroma (Figure 3o and p, and Figure 4d, $P<0.05$).

4.4. Expressions of eNOS and iNOS mRNAs in mouse uterus during early pregnancy

Different expression patterns for iNOS and eNOS

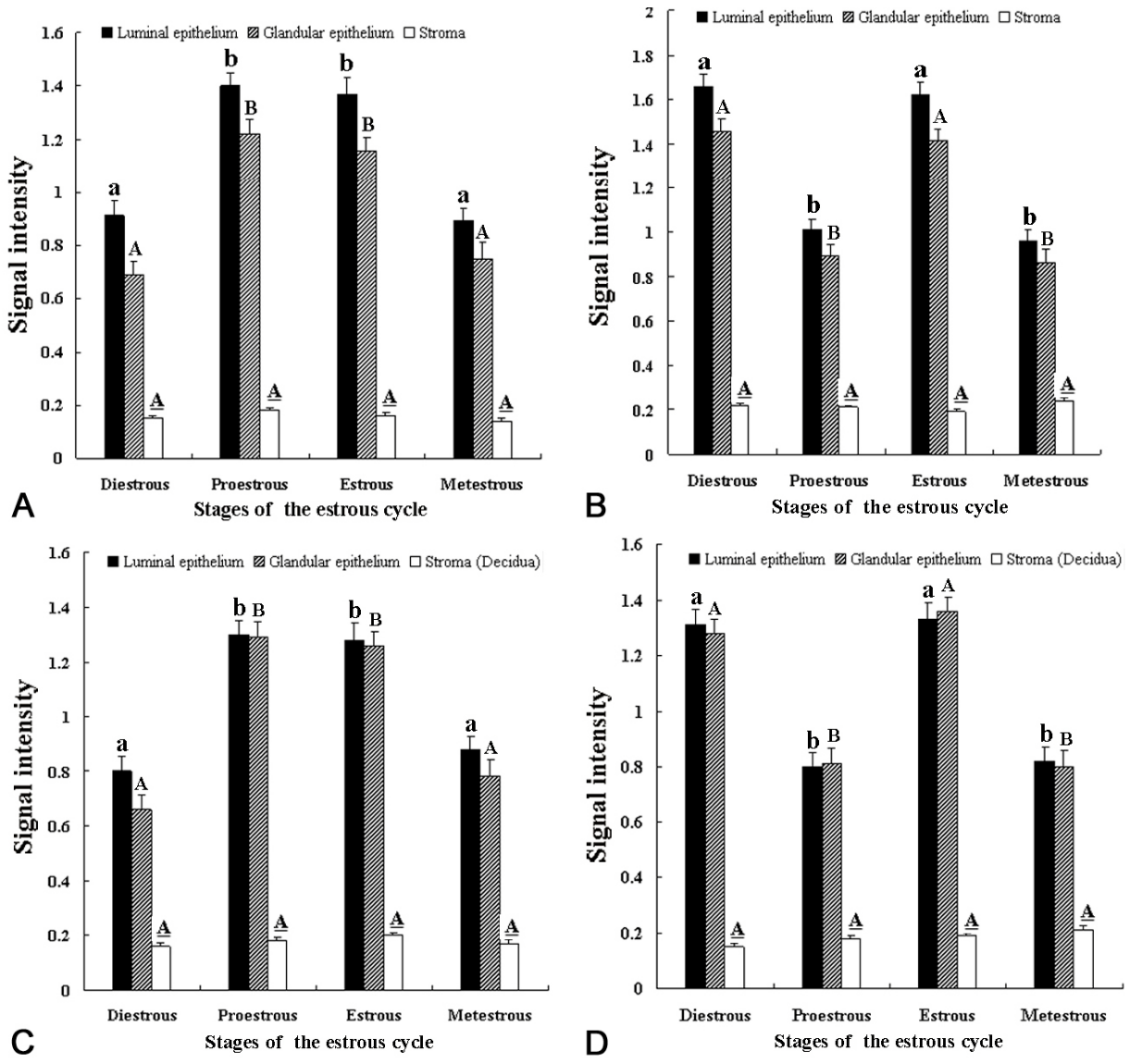

Figure 2. Quantitative analysis of *in situ* and immunohistochemical signals of eNOS and iNOS in mouse uteri during the estrous cycle. (a) *in situ* signals of eNOS mRNA during the estrous cycle; (b) *in situ* signals of iNOS mRNA during the estrous cycle; (c) immunohistochemical signals of eNOS protein during the estrous cycle; (d) immunohistochemical signals of iNOS protein during the estrous cycle. Bars carrying different letters (a and b, A, and B, or A and B) differ within each panel ($P<0.05$).

mRNA in peri-implantation uteri is noted in Figure 5-6. As shown in Figure 5, distinct signals for eNOS mRNA localized in the luminal and glandular epithelium on day 1 of pregnancy (Figure 5a). eNOS mRNA was also expressed in the luminal epithelium, glandular epithelium, and stromal bed on days 2 and 3 of pregnancy (Figure 5b and c). On days 4 and 5 of pregnancy, a distinct accumulation of eNOS mRNA was noted in the subepithelial stromal bed (Figure 5d and e), and with progression of the implantation process, eNOS mRNA accumulation occurred in both the mesometrial and antimesometrial decidua on days 6 and 7 of pregnancy (Figure 5f and g). No specific signals were detected when uterine sections were hybridized with sense probes (Figure 5h, Figure 6a, $P<0.05$).

On days 1 and 2 of pregnancy, intense iNOS mRNA signals were restricted to the uterine luminal epithelium and glandular epithelium (Figure 5i and j). By day 3 of pregnancy, iNOS mRNA was also present in uterine stroma, in addition to the luminal epithelium and glandular epithelium (Figure 5k). The distribution of iNOS mRNA was noted in the stroma on days 4 and 5 of pregnancy (Figure 5l and m). On days 6 and 7 of pregnancy, iNOS mRNA localized in the mesometrial and antimesometrial sides (Figure 5n and o). The expression of iNOS mRNA was increased at days 4, 5, and 6 of pregnancy. No specific signals were detected when uterine sections were hybridized with sense probes (Figure 5p, Figure 6b, $P<0.05$).

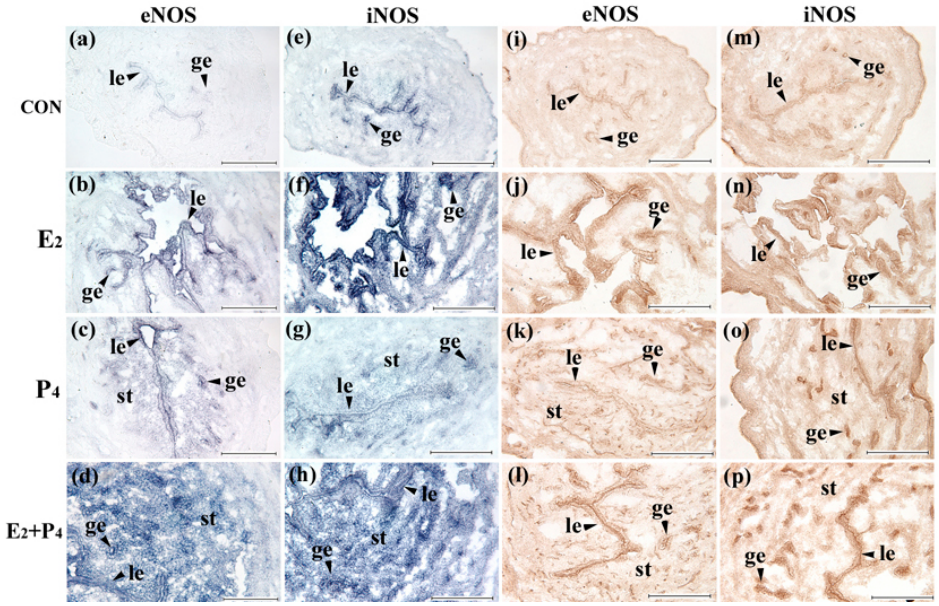

Figure 3. *In situ* and immunohistochemical localization of eNOS and iNOS in ovariectomized mouse uteri after E_2 or/and P_4 treatment. (a, e, i, m) the control group; (b-d) *in situ* localization of eNOS mRNA after E_2, P_4, and E_2+P_4 treatment; (f-h) *in situ* localization of iNOS mRNA after E_2, P_4, and E_2+P_4 treatment; (j-l) immunohistochemical localization of eNOS protein after E_2, P_4, and E_2+P_4 treatment; (n-p) immunohistochemical localization of iNOS protein after E_2, P_4, and E_2+P_4 treatment; le, luminal epithelium; ge, glandular epithelium; st, stroma; bar=200 μm.

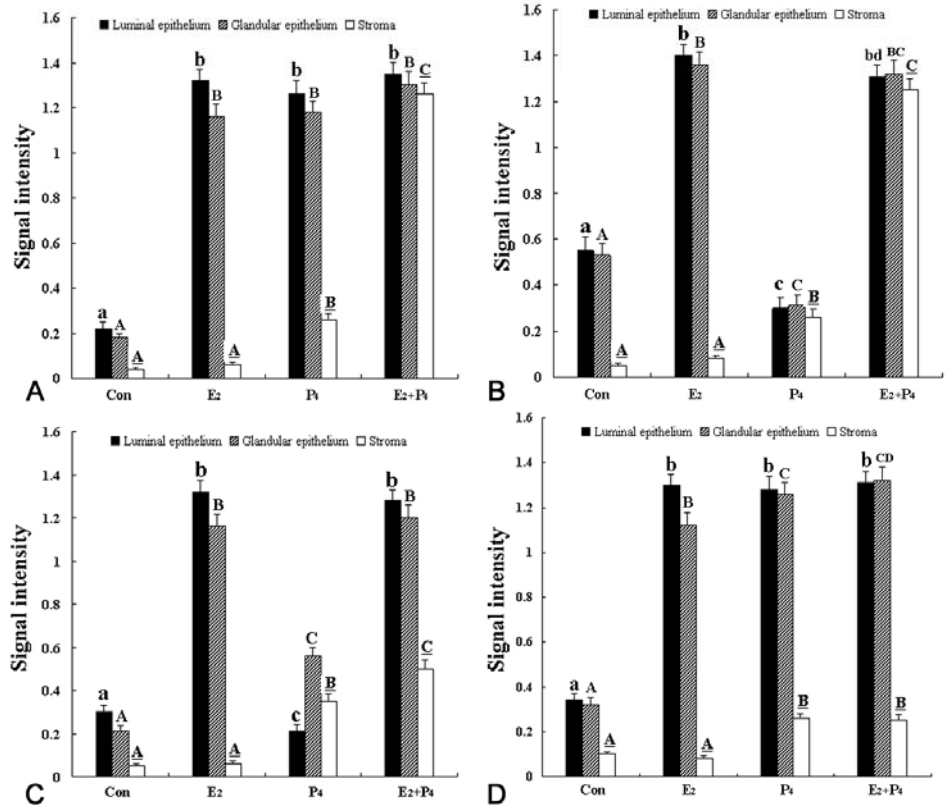

Figure 4. Quantitative analysis of *in situ* and immunohistochemical signals of eNOS and iNOS in ovariectomized mouse uteri after E_2 or/and P_4 treatment. (a) *in situ* signals of eNOS mRNA after E_2, P_4, and E_2+P_4 treatment; (b) *in situ* signals of iNOS mRNA after E_2, P_4, and E_2+P_4 treatment; (c) immunohistochemical signals of eNOS protein after E_2, P_4, and E_2+P_4 treatment; (d) immunohistochemical signals of iNOS protein after E_2, P_4, and E_2+P_4 treatment. Bars carrying different letters (a, b, c, and d; A, B, C, and D; or A, B, and C) differ within each panel (P<0.05).

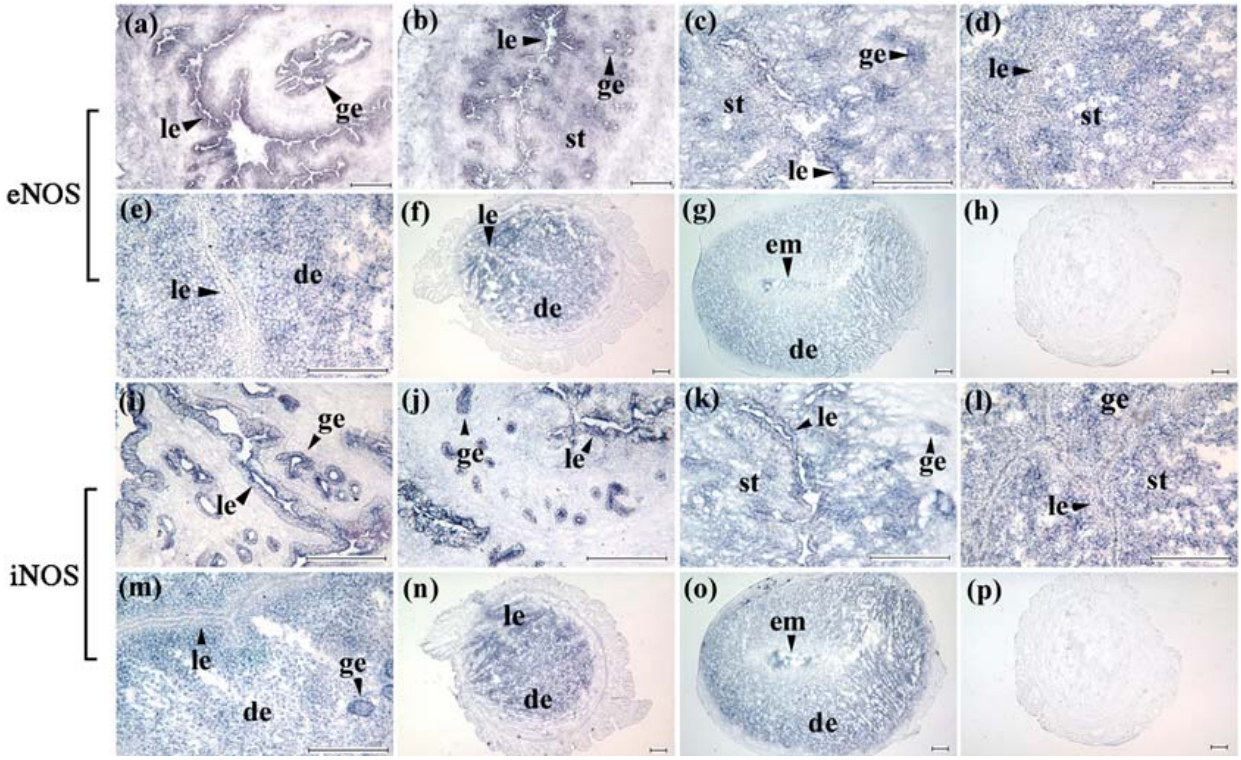

Figure 5. *In situ* hybridization of eNOS and iNOS mRNA in mouse uteri during early pregnancy. (a-g) the expression of eNOS mRNA on days 1-7 of pregnancy; (i-o) the expression of iNOS mRNA on days 1-7 of pregnancy; (h, p) negative controls; le, luminal epithelium; ge, glandular epithelium; st, stroma; em, embryo; de, decidua; bar=200 μm.

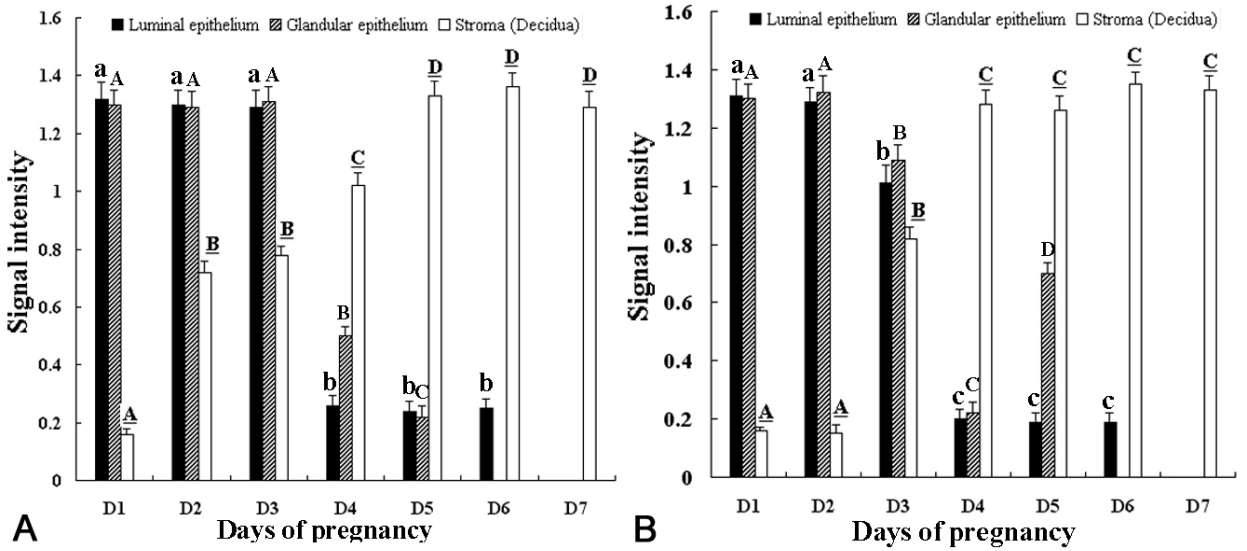

Figure 6. Quantitative analysis of *in situ* hybridization signals of eNOS and iNOS mRNA in mouse uteri during early pregnancy. (a) the expression of eNOS mRNA on days 1-7 of pregnancy; (b) the expression of iNOS mRNA on days 1-7 of pregnancy. Bars carrying different letters (a, b, and c; and d; A, B, C, and D; or A, B, C, and D) differ within each panel ($P<0.05$).

4.5. Expressions of eNOS and iNOS proteins in mouse uterus during early pregnancy

On days 1-3 of pregnancy, accumulation of eNOS and iNOS proteins occurred in the luminal epithelium and glandular epithelium (Figure 7a-c and Figure 7i-k). Strong signals for eNOS and iNOS proteins were detected in the glandular epithelium and luminal epithelium on days 4 and 5 of pregnancy, with weak signals localized in the subepithelial stroma (Figure 7d and e; Figure 7l and m). With the progression of implantation, eNOS and iNOS

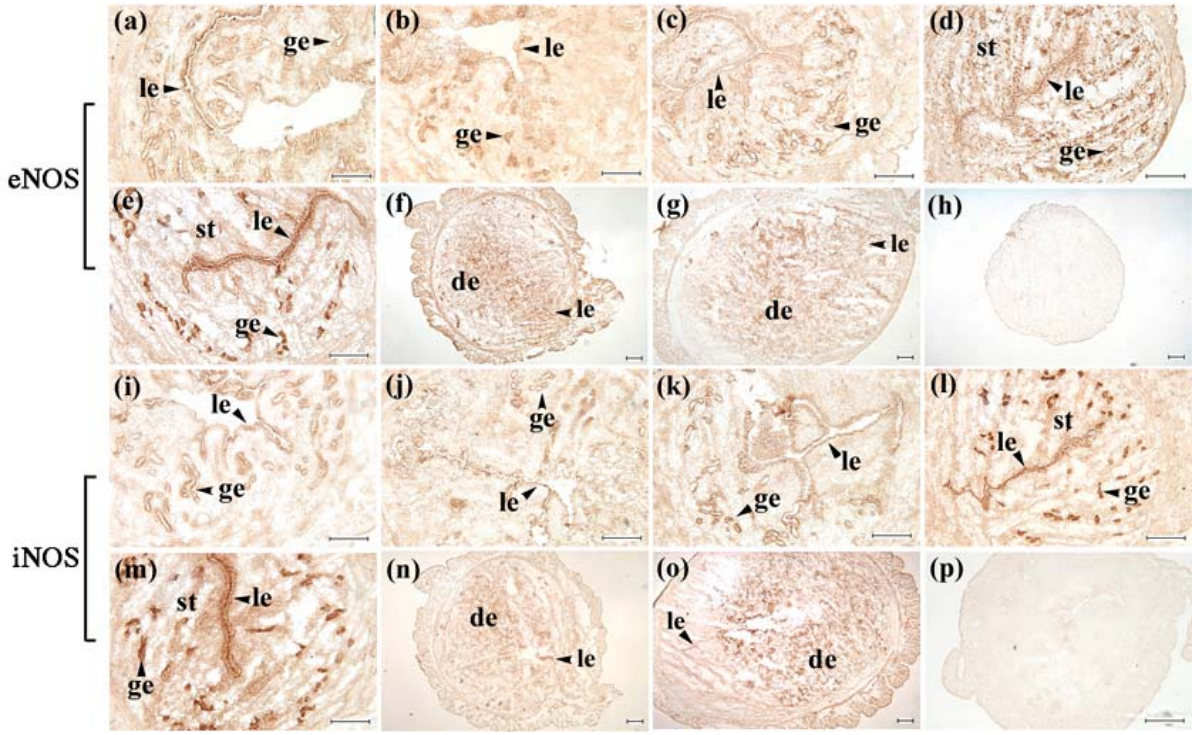

Figure 7. Immunohistochemical signals for eNOS and iNOS in mouse uteri during early pregnancy. (a-g) the expression of eNOS protein on days 1-7 of pregnancy; (i-o) the expression of iNOS protein on days 1-7 of pregnancy; (h, p) negative controls; le, luminal epithelium; ge, glandular epithelium; st, stroma; de, decidua; bar=200 μm.

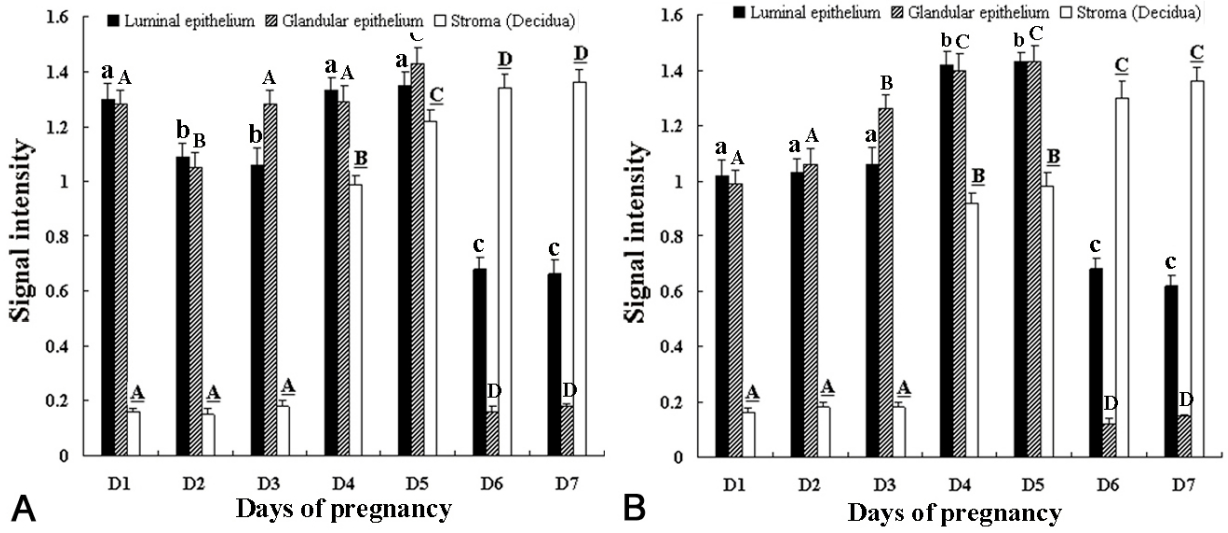

Figure 8. Quantitative analysis of immunohistochemical signals for eNOS and iNOS in mouse uteri during early pregnancy. (a) the expression of eNOS protein on days 1-7 of pregnancy; (b) the expression of iNOS protein on days 1-7 of pregnancy. Bars carrying different letters (a, b, and c; A, B, C, and D; or A, B, C, and D) differ within each panel ($P<0.05$).

proteins were localized in the decidualized stroma around the implanting embryo on day 6 (Figure 7f and n), and in the whole decidualized stroma at the implantation sites on day 7 (Figure 7g and o). No specific signals were detected in the negative control samples (Figure 7h and p). These results are summarized in Figure 8, $P<0.05$.

5. DISCUSSION

In an attempt to understand the roles of eNOS and iNOS in mouse uterus we have investigated the expression of eNOS and iNOS during the estrous cycle and early pregnancy, as well as the effects of ovarian steroid

hormones on uterine eNOS and iNOS expression, in ovariectomized mice using *in situ* hybridization and immunohistochemistry.

Studies have demonstrated that eNOS and iNOS are the primary isoforms expressed in the ovary and uterus (14), so we have focused on these two isoforms in this study. Previous reports have shown that human uterine epithelial cells display appreciable eNOS and iNOS signals during the menstrual cycle (15). In rats, the expression of iNOS was localized to the glandular epithelium and myometrial smooth muscle cells during the estrous cycle, and the level of iNOS was increased at proestrous and estrous (16). Here, we report that the expressions of eNOS and iNOS in mouse uteri were temporally and spatially restricted to the luminal and glandular epithelium during the estrous cycle. Previous evidence in conjunction with our results suggest that NO is involved in the maintenance of normal physiological endometrial function. In the present study, our results also revealed some differences in the expression patterns of eNOS and iNOS in mouse uteri. The expression of eNOS in the uterine epithelium was higher during proestrous and estrous than at diestrous and metestrous, while iNOS signals in the uterine epithelium were higher at diestrous and estrous than those seen during proestrous and metestrous. These changes are likely the result of variations in ovarian steroid hormone levels. These findings are also consistent with previous studies that demonstrated distinct expression patterns of eNOS and iNOS in human endometrium during the menstrual cycle (17-19). Thus, we suggest that the different expression patterns of eNOS and iNOS seen in the uterus imply slightly different functions for these isoforms during the estrous cycle. Studies performed in eNOS- and iNOS-deficient mice showed that the average estrous cycle was 4.8±0.2 days in wild-type (WT) mice, and there was no significant difference in cycle length between iNOS-deficient mice and WT mice; however, eNOS-knockout mice showed a significantly longer estrous cycle (6.6±0.6 days) relative to the WT and iNOS-knockout mice (7). The above evidence suggests a function for NOS/NO in normal estrous cycles, with uncoordinated functions for eNOS and iNOS during the estrous cycle. Despite the uniform functions of eNOS and iNOS pertaining to NO synthesis, their different expression patterns during the estrous/menstruous cycles lend support to the concept of specific functions. Further experiments should help elucidate the unique roles for each of these NOS isoforms.

The mammalian endometrium undergoes cyclic changes during the estrous cycle, which is regulated by ovarian steroid hormones. Therefore, ovarian steroid hormones might also regulate the expressions of eNOS and iNOS in mouse uteri during the estrous cycle. Indeed, we found that ovarian steroid hormones significantly regulated the expression patterns of eNOS and iNOS in the uterus of ovariectomized mice, and our results are consistent with previous observations in rats and humans (20, 21). In ovariectomized experiments, it is remarkable that treatment with progesterone does not recapitulate the events of diestrus, when progesterone is the dominant luteal hormone. It has been shown that progesterone up-regulates NOS gene expression and increases NO production in rat uterus (22). The mechanisms regulating uterine NOS expression during cycling uterus have not been fully elucidated. It is plausible that progesterone may exert a multiple effect during the estrous cycle in normal condition, and there are many downstream factors indirectly correlated to the estrous cycle. The establishment of a differentiated uterine environment supporting embryo implantation and development is primarily dependent on the coordinated effects of E_2 and P4 (23). Among the earliest endometrial responses to estrogen during implantation is a localized increase in vascular permeability, and the vasoregulatory role of NO is well-documented (24). In rats, the synthesis of NO is up-regulated in a gestational-dependent manner (25), and increased NO production in the uterus during pregnancy is a consequence of increased iNOS expression (26, 27). Furthermore, eNOS and iNOS proteins are increased in the uterus following implantation (28), and it is therefore plausible that NO might be intimately involved in the implantation process.

The highlight of the present investigation was that eNOS and iNOS were expressed in a specific temporal and spatial manner during the early implantation processes in mouse uteri. During the peri-implantation period, mRNAs and proteins of two NOS isoforms were present not only in the uterine epithelium, but also in the stroma, particularly in the primary decidual zone surrounding the implanting embryo on day 6 of pregnancy, and in the whole decidualized stroma (including the primary and secondary decidual zones) on day 7 of pregnancy. The stromal expressions of eNOS and iNOS were significantly higher during peri-implantation (days 4, 5 and 6 of pregnancy), and this result is consistent with the higher generation of NO in implanting mouse trophoblast (9). Novaro *et al.* demonstrated that E2 regulated NOS activity in rat uteri during implantation, and this increase in NOS activity was the result of a combined increase in the activity of both the Ca2+-dependent (cNOS) and the Ca2+-independent (iNOS) isoforms (29). The eNOS and iNOS mRNA expression in the luminal epithelium and glandular epithelium during pre-implantation was likely the result of an estrogen peak during preovulation, while peri-implantation eNOS and iNOS mRNA accumulation in the subepithelium stroma bed may be the integrated result of modest estrogen levels and high progesterone levels. The increase in iNOS and eNOS levels following implantation at the embryonic site may imply roles in tissue remodeling, immunosuppression, and vasoregulation. We also noticed that mRNA expression of the two isoforms was mainly located in the epithelium during the pre-implantation period, but localized to the stroma during the peri-implantation period; the proteins of the two isoforms were strongly expressed in the epithelium and stroma during the peri-implantation period. This concerted expression pattern between mRNA and protein during the pre-implantation period is likely the result of an intricate network regulating implantation and serving as a mediator in the generation of a successful pregnancy. It is also interesting that the expression of mRNA and protein do not concur during post-implantation period, the mRNA appears in the stroma, while the protein is most strongly

expressed in the epithelial components. It has been shown that a narrow range of NO concentrations, usually low, will stimulate or enhance some events in reproduction, but either a lack of NO or too much NO has negative consequences (30). Our results that the protein is the preponderant production during post-implantation also are the evidence of the narrow range in NO action. The difference in expression could reflect that the mechanism involving in the expression is different, or it may be due to an indirect effect of cytokines participated in the regulation of mRNA and protein. We also found that there are intense iNOS and eNOS proteins staining in epithelial cells, but no epithelial mRNA from days 4-6 of pregnancy, but little is known about the expression patterns of eNOS and iNOS in pregnancy. We supposed that some other process is responsible for it, or NOS synthesized in the stroma before migrating into the epithelium.

Embryo implantation is a complex process involving both maternal and embryonic signals. As an essential modulator of implantation, NO could be acting at multiple points to promote implantation. Previous reports have documented a functional role for iNOS in the maintenance of decidual cellular integrity and the development of uterine vasculature (31), and thus, iNOS might favor blastocyst attachment at implantation (32). On the other hand, it has also been reported that NO was able to induce programmed cell death (33) and extracelluar matrix degradation (34, 35), which are involved in embryo implantation (36, 37). Based upon our reports and other previous evidence, the endometrial roles of eNOS and iNOS are limited at this stage to educated speculation. The contributions of NOS to endometrial function will perhaps be further elucidated by the generation of double knockout mice that are deficient in both eNOS and iNOS.

In summary, the present results show that the expressions of eNOS and iNOS were localized in mouse uteri in specific temporal and spatial patterns during the estrous cycle and early pregnancy, and that the expression of two NOS isoforms was regulated by treatment with ovarian steroid hormones. Thus, increased NOS expression could be crucial in regulating embryo implantation.

6. ACKNOWLEDGEMENT

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