Effect of NO on the expression of VEGF and its receptors in mouse uterus during peri-implantation [*]

ZHANG Xuan[1] , WANG Hong-Mei[1] , QIAN Dong[1] , LIN Hai-Yan[1]
LIU Guo-Yi[1,2] , LI Qing-Lei[1] , ZHU Cheng[1] [**]

1. State Key Laboratory of Reproductive Biology , Institute of Zoology , Chinese Academy of Sciences , Beijing   100080 , China

2. Laboratory of Reproductive Endocrinology , Harbin Medical University , Harbin   150086 , China

Abstract   Nitric oxide (NO) is believed to play a pivotal role in embryo implantation. Successful embryo implantation depends upon the synchronized roles of hormones and a series of cytokines , and this event is accompanied by angiogenesis. As an angiogenic and vascular permeability factor , vascular endothelial growth factor (VEGF) is essential for endometrium development and placental vascular function during early pregnancy. The purpose of this study was to investigate the effect of NO on VEGF and its receptors , as well as the mechanism of NO during mouse implantation using intrauterine injection , *in situ* hybridization , and western blotting techniques. Nitric oxide synthase (NOS) inhibitor , N-nitro-L-arginine methyl ester (L-NAME) was administered with or without sodium nitroprusside (SNP) , NO donor , into one uterine horn on day 3 of pregnancy , and the contralateral uterine horn served as the control. We collected the uteri on days 5 , 6 , and 7 of pregnancy and examined the expression of VEGF and its receptors. The results showed that , compared with the control , the expression of VEGF and its receptors mRNAs declined in L-NAME-treated uteri during peri-implantation. Similarly , the western blotting results indicated that the protein levels of VEGF and its receptors decreased during peri-implantation. The L-NAME-mediated effect on the expression of VEGF and its receptors reversed when SNP was co-administered with L-NAME. These data suggested that inhibition of NO production regulated the expression of VEGF and its receptors during peri-implantation , which may have serious consequences on embryo implantation [*Acta Zoologica Sinica* 50 (1) : 55 - 61 , 2004].

Key words   Nitric oxide , Mouse , Embryo implantation , VEGF , Receptors

VEGF [*]

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1.   ,   100080
2.   ,   150086

NO   ,   Western blot
   (NO)   (VEGF)
   (D3)   (NOS)   N-   -L-   (L-NAME)   L-NAME
NO   (SNP)   ,   ;   D5 , D6   D7   VEGF
       mRNA   :   , L-NAME
VEGF   mRNA   ;   VEGF   ,   NO
   VEGF   NOS   NO   SNP
       , VEGF   mRNA   ,   NO
       VEGF   ,   [50 (1) : 55 - 61 ,
2004]

Nitric oxide (NO) is an intercellular signal molecule, synthesized by a family of enzymes known as nitric oxide synthase (NOS). In general, NOS isoforms are categorized into three types according to the different biochemical characteristics, that is, neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS) (Griffith and Stuehr, 1995); and the three NOS isoforms are generated by distinguishable genes. The production pathway of NO has been detected in a variety of cell types, including macrophages, endothelial cells, and -pancreatic cells (Oswald et al., 1994). NO was implicated in various physiological events, such as neuronal signaling, vasodilation, smooth muscle relaxation, and stimulation of immune responses (Bredt and Snyder, 1994). Recently, the roles of NO in the female reproductive system have been well established (Shukovski and Tsafriri, 1994). Specifically, the role for NO in embryo implantation has been demonstrated (Ota et al., 1999).

Successful embryo implantation requires the development of vascular network that facilitates maternal-fetal communication (Gordon et al., 1995). It is well known that the uterus undergoes important changes during implantation period, including increased vascular permeability and angiogenesis. Therefore, angiogenesis is a fundamental component in embryo implantation. The regulation network composed by various hormones, growth factors, and inhibitors modulated the formation of new vessels in embryo implantation and physiology condition (Ingber and Folkman, 1989). A number of vasodilatory substances were produced under the effect of oestrogen, such as acetylcholine, histamine, and VEGF, which were the inducer of endogenous NO (Jovanovic et al., 1994). As a homodimeric glycoprotein, vascular endothelial growth factor (VEGF) is a mitogen for endothelial cells and a potent inducer of angiogenesis, and plays an active role during early pregnancy (Athanassiades et al., 1998; Gerwins et al., 2000; Ghosh et al., 2000).

The family of VEGFs currently includes VEGF-A, -B, -C, -D, -E, and placenta growth factor (PlGF), and VEGF-A is usually known as VEGF. Alternate splicing of the VEGF transcript generates five isoforms, called $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$. VEGF works via two-tyrosine kinase family receptors that are c-fms-like tyrosine kinase (Flt-1) and fetal liver kinase-1 (Flk-1) (Shibuya et al., 1990; Millauer et al., 1993). It was known that VEGF mediated endothelial cells interaction and tubule formation by binding to Flt-1 whereas binding to Flk-1 caused endothelial cell differentiation and proliferation (Bernatchez et al., 1999).

The expression of VEGF and its receptors have been examined in mouse embryo during peri- and post-implantation (Shweiki et al., 1993). Numerous results converged to indicate a role for NO in physiological and pathological angiogenesis (Donnini and Ziche, 2002). As a signal molecule, NO played important roles in both angiogenesis and reproductive events (Jadeski and Lala, 1999). It might be deduced that NO was involved in implantation by this mechanism. It was also demonstrated that NO played important roles in VEGF-regulated angiogenesis and endothelial function, and VEGF could upregulate NOS expression by binding to its receptors. It was showed that VEGF enhanced the expression of eNOS through the tyrosine kinase-dependent pathway, an effect that may be important in the process of VEGF-induced angiogenesis (Kroll and Waltenberger, 1999; Bouloumie et al., 1999). The study in eNOS-/- mice suggested that eNOS acted downstream from VEGF in angiogenesis (Murohara et al., 1998). Studies in rats demonstrated that NOS was localized in the endometrium, and the production and activity of NOS increased at the implantation site (Saxena et al., 2000). In pregnant rats, iNOS was distributed within the decidua surrounding vessels and the ectoplacental cone, and eNOS was localized in vessels of the primary decidual zone adjacent to embryo (Purcell et al., 1999). The expression patterns of NOS suggested that NO might play important roles in implantation by regulating angiogenesis of implantation sites. Although the production of NO increased at the implantation site in uterus (Purcell et al., 1999; Novaro et al., 1997), its potential role in the regulation of VEGF and its receptors during embryo implantation has not been investigated. Previous work from our laboratory showed that intrauterine administration of the NOS inhibitor, N-nitro-L-arginine methyl ester (L-NAME), significantly reduced the number of successfully implanted embryos in pregnant mice (Zhang et al., 2002). These findings indicated that NO was required for successful embryo implantation. Intrauterine injection has several advantages in studying the mechanism of implantation, because it can reveal the local regulation on embryo implantation of test compounds, and the dose used is fewer (Cai et al., 2000). Therefore, the present investigation was undertaken to determine whether NO during peri-implantation period could regulate VEGF and its receptors, and elucidate the mechanism of NO in embryo implantation.

1　Materials and methods

1.1　Reagents

Tissue freezing medium was purchased from Triangle Biomedical Sciences, and restriction enzymes

were purchased from Promega. Trizol reagent was from Invitrogen. Bovine serum albumin (BSA), salmon sperm DNA (sDNA), deionized formamide, L-NAME, and sodium nitroprusside (SNP) were products of Sigma Chemicals. Digoxigenin (DIG) RNA labeling mix, anti-DIG-alkaline phosphatase, 4-nitro blue tetrazolium chloride (NBT), and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were products of Boehringer-Mannheim, Germany. Polyclonal rabbit anti-VEGF, anti-Flk-1, anti-Flt-1, monoclonal mouse anti-actin, as well as goat anti-IgG was purchased from Santa Cruz Biotechnology.

1.2 Animals and tissue collection

Adult mice of the outbred Kunming white strain (5 - 6 week age, 25 - 30 g weight) were provided by the Experimental Animal Center of Institute of Genetics, Chinese Academy of Sciences. Animals were bred at room temperature (RT) with controlled light cycles (12L 12D), and allowed free access to food and water.

Female mice were allowed to mate with male mice of the same strain (2 1). The day of vaginal plug was designated as day 1 of pregnancy. Pregnant mice were randomly divided into two groups, with 5 animals each. The animals were anaesthetized and injected with 0.2 mg L-NAME and 10 μg SNP in saline or 0.2 mg L-NAME in saline alone into one uterine horn, while the contralateral uterine horn received equal volume of saline (4μl) and served as the control (Zhang et al., 2002). On the morning of day 5, animals were anaesthetized and the implantation sites were defined by tail intravenous injection of 0.5 ml 1 % trypan blue 5 min before the dissection of the reproductive tract. On days 6 and 7, the implantation sites were visually distinct. Animals were sacrificed on the morning on days 5, 6, and 7 of pregnancy, and the uteri were excised, trimmed, and appropriate implantation sites were separated. The tissues were divided into two parts. One part was embedded in embedding medium for frozen tissue specimens and the other was snap-frozen in liquid nitrogen and stored at - 80 for protein extraction.

1.3 Probe labeling

The plasmids used in this study were kindly provided by Dr. S. K. Dey (Department of Molecular and Integrative Physiology, Kansas University Medical Center, KS), and Dr. Dong Qian (Institute of Zoology, Chinese Academy of Sciences, Beijing). The cDNA plasmids were linearized with appropriate restriction enzymes to provide template for antisense and sense riboprobes, respectively. Probe labeling was performed according to the instructions that came with the DIG RNA labeling mix.

1.4 In situ hybridization

In situ hybridization was performed according to the method of Braissant and Wahli (1998). with slight modifications. In brief, adjacent cryosections (10μm) of the implantation sites were cut at - 20 and mounted onto poly-L-lysine-coated slides. The sections were fixed for 15 min in freshly prepared 4 % paraformaldehyde in phosphate buffered saline (PBS, pH 7.2) and followed by incubated twice in PBS containing 0.1 % active diethylpyrocarbonate (DEPC) for 15 min each. After rinsing with 5 × SSC (1 × SSC is 0.015 mol/L sodium citrate and 0.15 mol/L NaCl) for 15 min, the sections were prehybridized with hybridization solution without probe (50 % deionized formamide, 5 × SSC, and 120μg/ml sDNA) for 2 h at 50 . The labeled probes (400 ng/ml) were added to the prehybridization solution and slides were hybridized at 50 for 18 h. Then the sections were incubated for 30 min in 2 × SSC, thereafter, washed in 2 × SSC for 1 h at 65 , 0.1 × SSC for 1 h at 65 . The slides were incubated with anti-DIG-alkaline phosphatase (diluted 1 3 000) for 2 h at RT. Color development was carried out using NBT-BCIP. To evaluate the background staining, DIG-labeled sense riboprobes were used for *in situ* hybridization under identical conditions to that used for antisense probes. The results were recorded with a SPOT digital camera system (Diagnostic Instruments, Inc., USA), and digital images were processed using Adobe Photoshop (version 5.5; Adobe, San Jose, CA, USA).

1.5 Western blotting

Protein was extracted according to the method provided by the Trizol reagent. Protein was added to sample buffer and boiled for 5 min. Approximately 50 μg protein/well was separated by SDS-PAGE. Gels were run on a Mini Protean vertical electrophoretic system (Bio-Rad) and then transferred to PVDF (polyvinylidene difluoride) membrane (100 V, 75 min). The membrane was washed in TBS (50 mmol/L Tris, 150 mmol/L NaCl, pH 7.5), then blocked with 3 % BSA at RT for 1 h, thereafter, incubated for 18 h at 4 with polyclonal rabbit anti-VEGF, anti-Flk-1, anti-Flt-1, and monoclonal mouse anti-actin. The membrane was incubated for 30 min at 37 with HRP-conjugated goat anti-IgG diluted 1 3 000. Finally, the membrane was washed three times in TBST (0.05 % Tween 20, TBS), 10 min each, and then processed by using NBT-BCIP detection system. The bands were analyzed using MetaView image analyzing system (4.50 version, USA). The intensity of the bands was corrected by comparison of actin levels.

1.6 Statistics

The results were expressed as means ± SE, and data were analyzed for statistical differences with Student t-test. A value of $P < 0.05$ was considered to be significant.

2 Results

2.1 In situ **hybridization results of VEGF and its receptors mRNAs after treated with L-NAME/L-NAME + SNP**

The results of *in situ* hybridization showed that, on both L-NAME-treated uteri and corresponding control uteri, clear signals for VEGF mRNA were detected on days 5, 6, and 7 of pregnancy (Fig. 1). The level of VEGF mRNA was weak on day 5 of pregnancy. With the progression of decidualization, the signals of VEGF mRNA were primarily localized in the decidual zone immediately surrounding the blastocyst on day 6 and day 7 of pregnancy. Compared with the control, VEGF mRNA level was significantly decreased in L-NAME-treated uterine horns on days 6, and 7 of pregnancy (Fig. 1 A - F).

Flk-1 and Flt-1 mRNAs were visualized in uteri on days 5, 6, and 7 of pregnancy, and the expression of Flk-1 and Flt-1 mRNAs were decreased in the L-NAME-treated uteri compared with that of the control uteri (Fig. 2 A - F, Fig. 3 A - F). In L-NAME + SNP treated uteri, the mRNA expression of VEGF and its receptors did not change compare with that of the control uteri on days 5, 6, and 7 of pregnancy (Fig. 1 G - L, Fig. 2 G - L, Fig. 3 G - L).
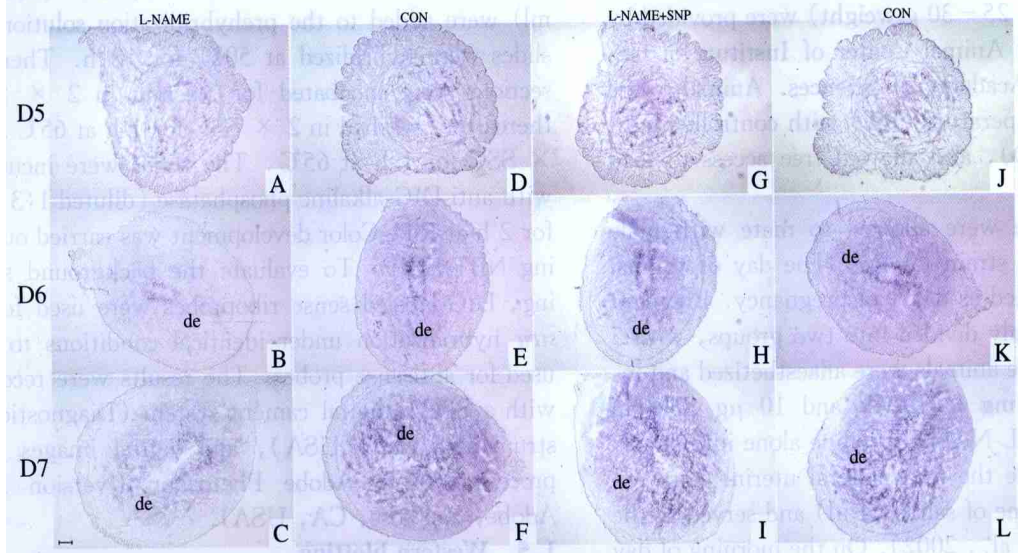

Fig. 1   In situ **hybridization localization of VEGF mRNA in mouse uterus on days 5, 6, and 7 of pregnancy under L-NAME/L-NAME + SNP**
L-NAME-treated uteri (A - C) and the corresponding control uteri (D - F). L-NAME + SNP-treated uteri (G - I) and the corresponding control uteri (J - L) were collected from day 5 (A, D, G, J), day 6 (B, E, H, K), and day 7 (C, F, I, L) of pregnancy.
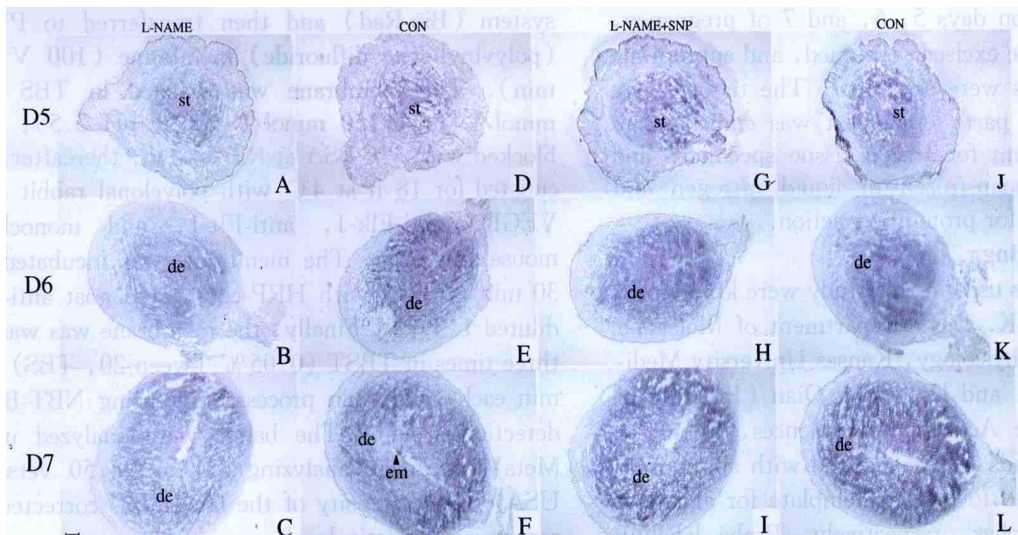CON: the corresponding control group. de: decidua. Bar = 200μm.

Fig. 2   In situ **hybridization localization of Flk-1 mRNA in mouse uterus on days 5, 6, and 7 of pregnancy under L-NAME/L-NAME + SNP**
L-NAME-treated uteri (A - C) and the corresponding control uteri (D - F). L-NAME + SNP-treated uteri (G - I) and the corresponding control uteri (J - L) were collected from day 5 (A, D, G, J), day 6 (B, E, H, K), and day 7 (C, F, I, L) of pregnancy.
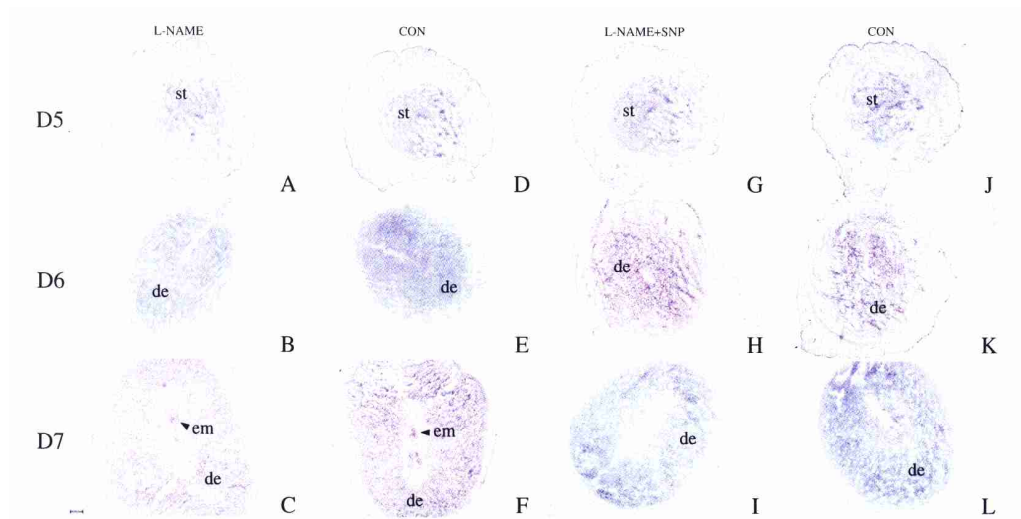CON: The corresponding control group. st: stroma. em: embryo. de: decidua. Bar = 200μm.

Fig. 3   In situ **hybridization localization of Flt-1 mRNA in mouse uterus on days 5 , 6 , and 7 of pregnancy under L-NAME/L-NAME + SNP**

L-NAME-treated uteri (A - C) and the corresponding control uteri (D - F). L-NAME + SNP-treated uteri (G- I) and the corresponding control uteri (J - L) were collected from day 5 (A , D , G, J) , day 6 (B , E , H , K) , and day 7 (C , F , I, L) of pregnancy. CON: The corresponding control group. st : stroma. em : embryo. de : deciduas. Bar = 200 μ m.

2. 2   Western blotting results of VEGF and receptors proteins after treated with L-NAME/L-NAME + SNP

The protein extracts of uteri treated with L-NAME/L-NAME + SNP and the corresponding control uteri on days 5 , 6 , and 7 of pregnancy were subjected to western blotting in order to evaluate the protein levels of VEGF and its receptors during peri-implantation period. Compared with the corresponding control , the expression of VEGF and its receptors proteins declined markedly on days 5 , 6 , and 7 of pregnancy after treatment with L-NAME. Statistical analysis showed that the levels of VEGF and its receptors were significantly decreased on days 5 , 6 , and 7 of pregnancy after treated with L-NAME. In L-NAME + SNP treated group , the expression of VEGF and its receptors were not significantly different from the corresponding control on these time points examined (Fig. 4 , Table 1).

Table 1   Quantification of VEGF, Flk-1 , and Flt-1 proteins in mouse uterus treated with L-NAME alone or co-administered with SNP on days 5 (D5) , 6 (D6) , and 7 (D7) of pregnancy (n = 5)

		L-NAME	CON	L-NAME + SNP	CON
D5	VEGF	0.153 ± 0.025 **	0.290 ± 0.030	0.270 ± 0.030	0.280 ± 0.025
	Flk-1	0.250 ± 0.021 **	0.345 ± 0.028	0.336 ± 0.024	0.326 ± 0.031
	Flt-1	0.175 ± 0.026 **	0.248 ± 0.029	0.260 ± 0.027	0.251 ± 0.032
D6	VEGF	0.150 ± 0.026 **	0.320 ± 0.030	0.350 ± 0.035	0.323 ± 0.032
	Flk-1	0.240 ± 0.024 **	0.350 ± 0.029	0.340 ± 0.030	0.330 ± 0.029
	Flt-1	0.200 ± 0.026 **	0.330 ± 0.032	0.317 ± 0.036	0.340 ± 0.026
D7	VEGF	0.380 ± 0.025 *	0.490 ± 0.028	0.482 ± 0.025	0.468 ± 0.026
	Flk-1	0.327 ± 0.022 *	0.504 ± 0.025	0.510 ± 0.028	0.516 ± 0.030
	Flt-1	0.260 ± 0.021 **	0.389 ± 0.026	0.370 ± 0.024	0.359 ± 0.029

The protein of VEGF , Flk-1 , and Flt-1 detected by western blotting were quantified by MetaView image analyzing system. The intensity of the bands was corrected by comparison of actin levels. Numbers are means ± SE of five experiments. Compared with its corresponding control : * $P < 0.05$; ** $P < 0.01$.
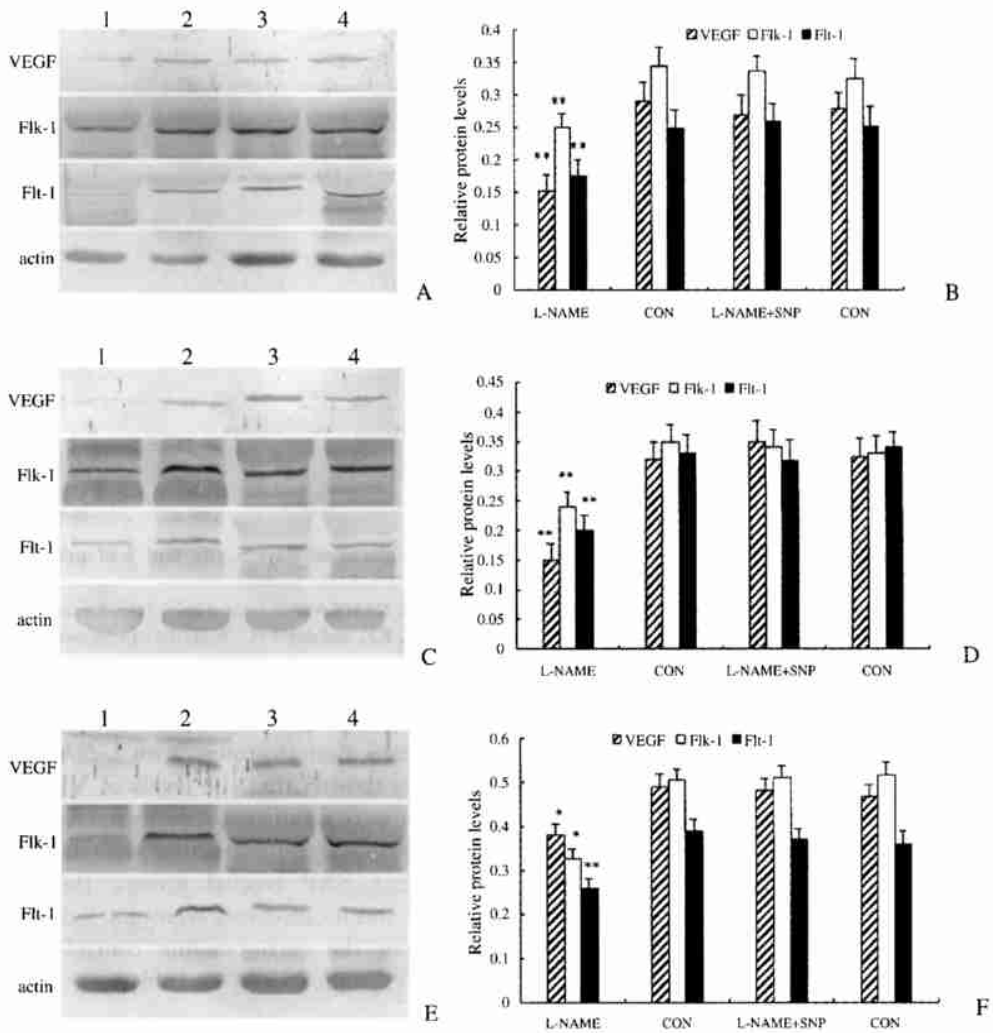
Fig. 4   Detection of VEGF and its receptors proteins in mouse uteri treated with L‑NAME alone or co‑administered with SNP
A , C and E: Western blotting of mouse uteri collected on days 5 , 6 , and 7 of pregnancy. Lanes 1 and 2 : L‑NAME‑treated uterine horn and the corresponding control uterine horn. Lanes 3 and 4 : L‑NAME + SNP‑treated uterine horn and the corresponding control uterine horn. B , D and F: Proteins were quantified by MetaView image analyzing system. Values are means ± SE from five experiments. Compared with its corresponding control , * represents $P < 0.05$, and ** represents $P < 0.01$.

3   Discussion

Our results demonstrated that the expression of VEGF and its receptors was declined after treatment with L‑NAME during peri‑implantation period , and L‑NAME‑induced changes of VEGF and its receptors expression was rescued by simultaneously treating pregnant mice uteri with SNP and L‑NAME. Our results suggested that NO was involved in implantation by regulating VEGF and its receptors expression. Another study showed that increased NOS expression enhanced the generation of VEGF (Dulak et al. , 2000). Furthermore , VEGF positively regulated NO production in endothelial cells. In addition , NO attributed to the increasing of microvascular permeability mediated by VEGF (van der Zee et al. , 1997). All the results showed that there was mutual regulation between NO and VEGF , and the positive inter-

action between endogenous NO and VEGF will undergo transformation under distinct physiological and pathological status. It is plausible that the exquisite regulation between NO and VEGF may have implications on modulation diverse physiology events. In the mouse uterus , the declined expression of VEGF may inhibit the angiogenesis after treatment with L‑NAME during implantation , and then the embryo implantation was suppressed.

NO was an upstream modulator in VEGF and its receptors‑regulated angiogenesis , and NO could regulate the expression of VEGF receptors (Bussolati et al. , 2001). Our *in vivo* study supported this view. Although the expression of VEGF and receptors was regulated by L‑NEME in mouse uteri , it was not clear that the regulation of Flk‑1 and Flt‑1 by L‑NAME was directed or indirect by decreased VEGF expression. The regulation mechanism between NO

and V EGF is also presently unclear. In endothelial cells , V EGF increased eNOS expression and NO production via activation of the Flk-1 and a downstream protein kinase C signaling pathway (Shen et al. , 1999) , which showed that there was also mutual regulation between NO and the V EGF receptor. Future studies should be conducted to decipher the signaling pathways of regulation between NO and VEGF/ receptors.

In summary , our results showed that L-NAME could regulate V EGF and the expression of its receptors during peri-implantation period. This observation suggested that NO participated in embryo implantation through V EGF-induced angiogenesis. Future investigation will focus on elucidating the mechanism of NO in embryo implantation. This may provide a new idea for understanding the signaling network pathways of implantation , and will help decipher the mechanism of implantation and develop contraceptive ways.

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