Regulation of embryo implantation by nitric oxide in mouse

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Abstract **Intrauterine injection and zymography were used to investigate the effect of nitric oxide (NO) on embryo implantation in mice. On day 3, one uterine horn of female pregnant mice was injected intraluminally with various doses of nitric oxide synthase (NOS) inhibitor, N-nitro-L-arginine methyl ester (L-NAME), while the contralateral horn served as control. Animals were sacrificed by cervical dislocation on day 7 of gestation, and the number of implanted embryos in each horn was calculated. The results showed that lower doses (0.05 mg L-NAME) did not inhibit implantation significantly ($P > 0.05$), but high doses (0.2 mg L- NAME) resulted in a significant reduction in the number of implanted embryos ($P < 0.05$). Co-administration of SNP, a generator of NO, with L-NAME would reverse the anti-implantation effect of L-NAME. To further understand the precise mechanism of NO in implantation, matrix metalloproteinase (MMPs) activities were detected by gelatin zymography. The reduction in the number of implanted embryos in 0.2 mg L-NAME treated group was associated with decreased MMP-9 activity but a stable MMP-2 activity. The activities of MMP-2 and MMP-9 were not changed in L-NAME and SNP treated group. These data suggest that NO acts as a mediator to regulate the activity of MMP-9, and facilitates embryo implantation.**

Keywords: mouse, nitric oxide, nitric oxide synthase, embryo implantation, matrix metalloproteinases.

As an important paracrine signal molecule, NO is involved in various physiological responses, such as regulating blood flow, thrombus forming, neurotransmission, and macrophage cytotoxicity[1]. In reproductive organs, NO is implicated in several reproductive events[2]. NO is synthesized by a family of enzymes known as nitric oxide synthase (NOS) in a variety of tissues and cells. NOS isoforms are categorized into three types: neuronal NOS (nNOS), inflammatory NOS (iNOS), and endothelial NOS (eNOS) according to the cell localization, properties, and catalytic mechanism of NOS[3].

Embryo implantation begins at special time after ovulation. Successful embryo implantation depends upon the synchronized development of both the invasiveness of embryo and the receptivity of uterine endometrium. This process is highly controlled by a series of factors[4]. During implantation period, endometrium undergoes decidualization, and manifests the maximal uterine receptivity that provides a suitable environment for embryo implantation. This event is accompanied by extensive degradation and remodeling of extracellular matrix (ECM). Three enzyme families, including plasminogen activators (PAs), cathepsin, and matrix metalloproteinase (MMPs) are responsible for the degradation of ECM. MMPs are the main elements for degrading ECM in implantation[5—7]. Studies in rats demonstrate that NOS is localized in endometrium, and the production and activity of NOS are increased at the implantation site. These findings indicate that NO is required for successful embryo implantation[8, 9]. In placenta, NO plays a role in placental function by regulating MMP-9 activity [10], but in embryo implantation, the relationship between NO and MMPs is not clear.

In the present work, we used mouse intrauterine injection and gelatin zymography to further investigate the effect of NO in implantation, and tried to elucidate the mechanism of NO in embryo implantation.

1 Materials and methods

(ⅰ) Reagents. N-nitro-L-arginine methyl ester (L-NAME), sodium nitroprusside (SNP) and gelatin were products of Sigma. NOS detect assay kit was purchased from the Institute of Biomedicine Engineering of Nanjing Juli, China.

(ⅱ) Animals. Adult mice of the outbred Kunming white strain (12-week age, 25—30 g weight) were provided by the Experimental Animal Center of the Institute of Genetics, the Chinese Academy of Sciences. Animals were bred at room temperature (about 25℃) in a constant photoperiod (light∶dark cycle, 12L∶12D) and allowed free access to food and water.

(ⅲ) Intrauterine injection. Female mice were allowed to mate with the same strain male mice (2∶1). Pregnancy was confirmed by the presence of vaginal plug on the next morning and this was termed day 1 of pregnancy. Mice were randomly distributed into 3 experimental groups with 8—10 animals each. On day 3 of gestation, these mice were anaesthetized and injected with test compounds (diluted in 4 μL 0.9% saline) into one uterine horn, while the contralateral horn received 0.9% saline (4 μL) and served as a control, ⅰ) treated with 0.05 mg L-NAME; ⅱ) treated with 0.2 mg L-NAME; ⅲ) treated with 0.2 mg L-NAME and 10 μg SNP. On day 7 of gestation, the treated animals were killed and the number of implanted embryos was counted. The uteri were collected and stored at −80℃ until use.

(ⅳ) Assay of NOS activity. NOS activity was measured using a NOS detect assay kit according to the kit guide, and was expressed in unit of enzyme activity per microgram of protein. In brief, mice were treated with L-NAME on day 3 of gestation using intrauterine injec-

tion, and the treated and control uterine horns were collected on day 5 of gestation. Uterine tissue (100 mg) was homogenized in 0.9 mL 0.9% saline. The homogenates were centrifuged for 5 min at 10000 g, and 50 μL supernatant was applied to assay the activity of NOS.

(v) Gelatin zymography. Protein extraction was performed according to the method provided by the Trizol reagent (Gibco BRL life Technologies Inc.). Protein extracts were mixed with sample buffer (0.25 mol/L Tris-HCl, 40% Glycerol, 0.04% bromophenol blue, 8% SDS, pH 6.8), then incubated for 30 min at 37℃. Protein samples were subjected to electrophoresis in a 10% polyacrylamide gel containing 0.5 mg/mL gelatin. After electrophoresis, the gel was treated twice for 30 min each time in 2.5% Triton X-100 (2.5% Triton X-100, 0.05 mol/L Tris- HCl, pH 7.5) and incubated for 18 h in a calcium buffer (0.05 mol/L Tris-HCl, 0.2 mol/L NaCl, 0.01 mol/L $CaCl_2$, 1% Triton X-100, 1 μmol/L $ZnCl_2$, pH 7.5). Gel was stained with 0.2% Coomassie brilliant blue R-250. MMPs activities were visualized as clear bands after destaining with 10% acetic acid. Activities of MMP-2 and MMP-9 detected by gelatin zymography were quantified by computer-aided densitometry (Personal Densitometer SI; Molecular Dynamics Inc., Sunnyvale, CA)[11].

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

2 Results

(i) Intrauterine injection. Fig. 1(a) summarized the results of injection of L-NAME alone or in combination with SNP into a uterine horn of pregnant mice on day 3. Injection of 0.05 mg L-NAME decreased the number of implanted embryos, but the difference was not significant ($P > 0.05$) compared with control. The number of implanted embryos in the 0.2 mg L-NAME treated horn was dramatically decreased ($P<0.05$) and that in L-NAME-SNP treated uterine horn was not significantly different from its control. Fig. 1(b) showed photograph of uterine horns on day 7 of gestation with diminished number of implanted embryos in the uterine horn injected with 0.2 mg L-NAME.

(ii) Assay of NOS activity. Fig. 2 shows that the activity of NOS in the uterus was significantly suppressed after injection of 0.2 mg L-NAME ($P < 0.01$).

(iii) Gelatin zymography. Figs. 3 and 4 show the activities of MMP-2 and MMP-9 after administration of L-NAME with or without SNP. Three bands of gelatin activity at 92, 72 and 64 kD were detected, which are consistent with latent MMP-9 and the latent and active forms of MMP-2 respectively. Compared with control, the band of 92 kD (MMP-9) was weak, but the bands of 72 and 64 kD were not changed significantly in 0.2 mg L-NAME treated group. Activities of MMP-2 and MMP-9 detected by gelatin zymography were quantified by computer-aided densitometry, and the data were analyzed for statistical difference. The results showed that the activity of MMP-9 was significantly decreased ($P < 0.01$), but there was no difference in MMP-2 activity between the control and treatment groups (fig. 3). To ensure that the change of MMPs activities was due to the inhibited NO

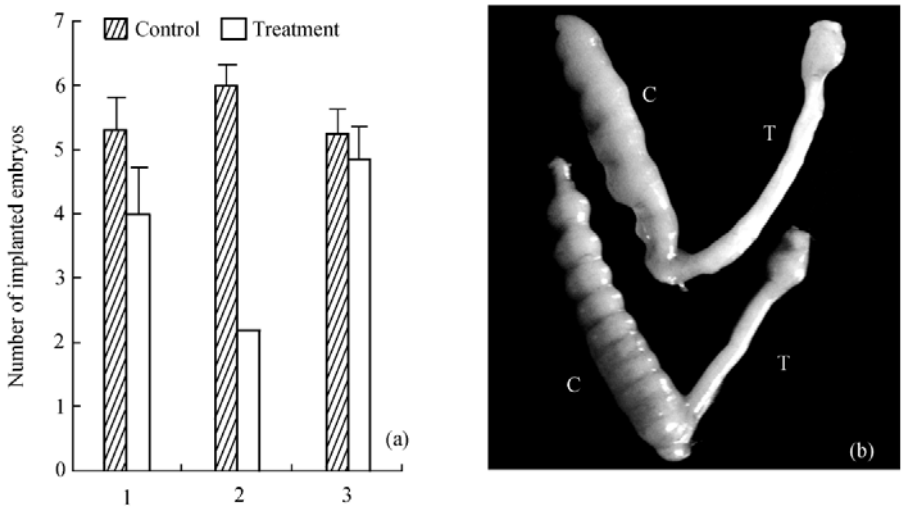

Fig. 1. Effect of intrauterine injection of L-NAME, a NOS inhibitor on embryo implantation in mouse, $n = 6$ animals in each group. (a) Compared with control, ** $P < 0.01$. (b) Photograph of the uterine horns on day 7 of gestation. It can be observed that there is one embryo implanted in the uterine horn injected with L-NAME.

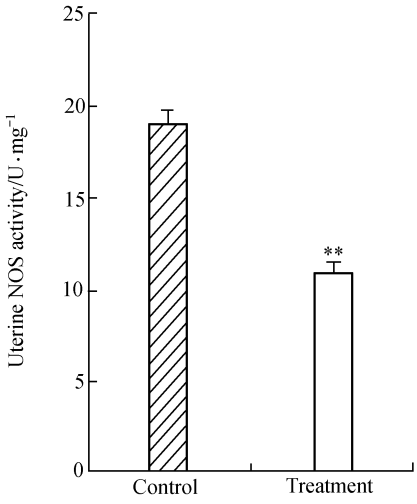

Fig. 2. Uterine NOS activity in day 5 of pregnancy after administration of 0.2 mg L-NAME. ** represents very significant difference compared to the control ($P < 0.01$). $n = 6$ animals in each group.

production after injection of L-NAME, the activities of MMPs in L-NAME and SNP treated uterus were detected. The results showed that the activities of MMP-2 and MMP-9 were not different in L-NAME-SNP treated groups ($P > 0.05$) compared to control (fig. 4).

3 Discussion

As a multifunctional molecule, NO is involved in embryo implantation by various ways. NO is known to increase capillary permeability by affecting the production of cGMP. Moreover, NO increases the production of prostaglandin(PGs), which is an important factor in regulating vascular permeability and decidualization[9]. NO may regulate embryo implantation by mediating apoptosis[12]. Till now, detailed mechanisms of NO in embryo implantation are still poorly understood. We have investigated the role of NO in embryo implantation using intrauterine injection and gelatin zymography for understanding the mechanism of NO in implantation. Intrauterine injection method is a potential model to reveal the local regulation on embryo implantation of test compounds, and the dose used is fewer[13].

L-NAME, an inhibitor of NOS, that converts L-arginine to NO, inhibits the production of NO. In mice, L-NAME was administered at various doses on day 3 of gestation. The results showed that 0.2 mg L-NAME was effective in preventing implantation, but 0.05 mg L-NAME was not. To ensure that the activity of NOS was inhibited after injection of L-NAME, we detected NOS activity in the uterus on day 5 of gestation. The results showed that NOS activity of treated uterine horn was significantly decreased. When SNP, a donor of NO, was administered in combination with L-NAME, the number of implanted embryo was reversed. The above data suggest that NO plays an important role in implantation.

Embryo implantation is a highly regulated process. Synchronous interaction of various factors is essential for establishment of pregnancy. A distinct event occurred in implantation is the ECM remodeling. MMPs are the critical enzymes in ECM degradation. MMPs are makers of invasive embryo in that the expression of MMP is consistent with the maximal invasive ability of embryo[14]. Now, the studies on MMPs have become the focus in embryo implantation. In diverse species, the specific expression of MMP-2 and MMP-9 exists in uterus during embryo implantation[11,15], suggesting an important role of MMP in implantation. In mice, the temporal and spatial expression of MMP-2 and -9 is dynamic. On day 4.5 of gestation, weak signals for MMP-9 mRNA were detected in trophoblast giant cells. The expression of MMP-9 mRNA was significantly increased on day 5.5—8.5 of gestation, but then disappeared. These results indicate that MMP-9 plays a role in trophoblast invasiveness. MMP-2 mRNA is primarily expressed in the endometrial stroma in implantation period. On day 6 of gestation, signals were primarily in the secondary decidual zone. On day 7—8 of gestation, MMP-2 mRNA was localized at the stroma adjacent to myometrium[16,17]. This data showed that MMP-2 participate in decidualization. The above evidence indicates that MMPs are involved in the invasion of trophoblast cells and decidualization. MMPs, as a downstream regulator involved in embryo implantation, its activity and gene transcription were modulated by many factors[18—20]. NO might be involved in implantation by regulating the tissue remodeling[8, 9], but the mechanism of NO in implantation has yet to be established.

Our data showed that both the number of implanted embryos and activity of MMP-9 were decreased after injection of L-NAME. SNP reversed the anti-implantation effect of L-NAME, at the same time, the activity of MMP-9 become normal. Taken together, these results suggest that NO is involved in embryo implantation by local regulating the activity of MMP-9. In our study, the activity of MMP-2 was constant, which suggested that the role of two types of MMPs was different in implantation. MMP-9 may be critical in embryo implantation. Further research will focus on the regulation of NO in activity and transcription of MMPs on different days during the peri-implantation phase of pregnancy, allowing us to clarify the function of NO during implantation.

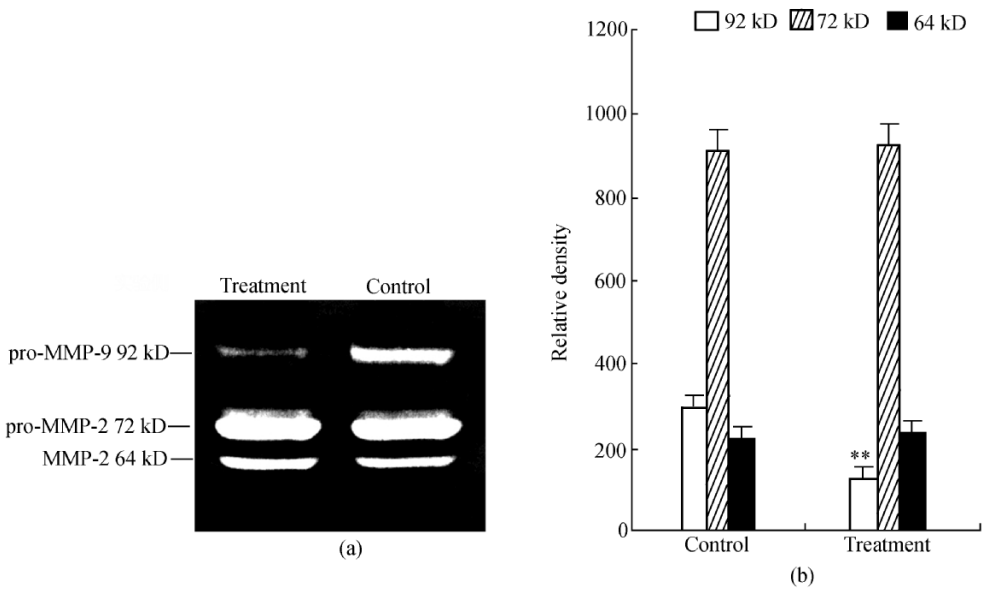

Fig. 3. Uterine MMP-2 and MMP-9 activities after administration of 0.2 mg L-NAME. ** represents very significant difference compared to the control ($P < 0.01$). $n = 6$ animals in each group.

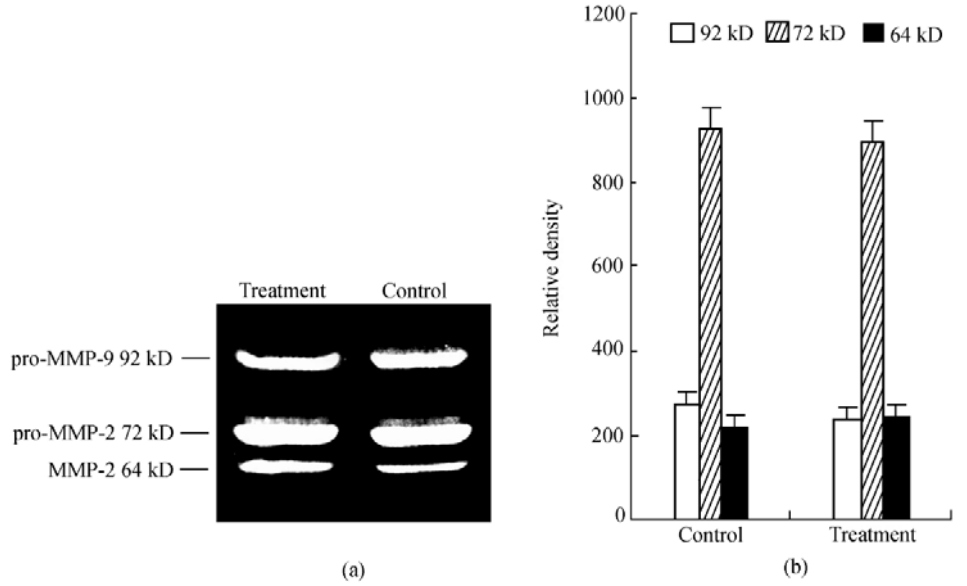

Fig. 4. Uterine MMP-2 and MMP-9 activities after co-administration of 0.2 mg L-NAME and 10 μg SNP. The activities of MMP-2 and MMP-9 were not different from the control ($P > 0.05$), $n = 6$ animals in each group.

Overall, our results indicate that NO, as a paracrine factor, participates in embryo implantation by regulating the activity of MMPs. However, much remains to be learned about its precise function. An enhanced understand of the function of NO will not only help decipher the signaling pathways regulating embryo implantation, but also offer a possible way for birth control.

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