Original Research

Molsidomine and *N*-omega-nitro-*L*-arginine methyl ester inhibit implantation and apoptosis in mouse endometrium[1]

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KEY WORDS nitric oxide; apoptosis; pregnancy rate; endometrium

ABSTRACT

AIM: To investigate the possible effect of nitric oxide on receptivity and apoptosis of mouse endometrium and the possible pathway. **METHODS:** Female pregnant mice were treated with either molsidomine, a generator of nitric oxide (NO), or *N*-omega-nitro-*L*-arginine methyl ester (*L*-NAME), an inhibitor of NO synthase. The pregnancy rates of each group were calculated; 3'-end-labeling was used to detect DNA fragmention of apoptotic cells; immunohistochemistry, *in situ* hybridization, and Western blot were applied respectively to estimate expression levels of Fas/FasL proteins and mRNA. **RESULTS:** The pregnancy rate in the drug treated group was reduced in a dose-dependent manner; apoptosis, Fas protein and mRNA levels in the endometrium of drug treated mice were correlatively decreased during the peri-implantation period. **CONCLUSION:** The decreased pregnant rate in mice by abnormal levels of nitric oxide may be brought about by inhibiting the normally occurrence of apoptosis in the receptive endometrium.

INTRODUCTION

The endometrium in peri-implantation period is critical for early embryo development. Trophoblast-uterine interaction involves several progressive stages under the action of luteal hormones during implantation process, including uterine stroma transforming into enlarged decidual cells[1] and the uterine glands changing their secretory patterns[2] and accompanied by remarkable apoptosis. However, the molecular mechanism of implantation is not clearly defined yet.

Nitric oxide (NO) is a small, but potent molecule which plays an important role as a major paracrine mediator in various biological processes, such as vasodilatation, relaxation of nonvascular smooth muscle, inhibition of platelet aggregation, leukocyte adhesion and rolling, neurotransmission, and mediation of cytotoxic macrophage action[3-5]. *L*-arginine is converted by the enzyme nitric oxide synthase (NOS) into NO. Three well-known isoforms of NOS, neuronal (nNOS), endothelial (eNOS), and cytokine-inducible NOS (iNOS), have been identified. NO may be an important regulator in several processes related to implantation. In uterus, nitric oxide is involved in relaxation of myometrium and vessels[6] and induction of stromal edema[7]. It is known that all three NOS isoforms are present within the endometrium of human and rodents. eNOS localizes in

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human glandular epithelium and microvascular endothelium, during the luteal phase, and iNOS mainly in the endometrial epithelium and decidualized stromal cells[8]. In rodents, both iNOS and eNOS are highly up-regulated in the implantation sites[9], but the detailed function of NO during implantation processe is still not fully understood.

Abnormal level of nitric oxide is often accompanied with infertility and miscarriage states, such as endometriosis[10], fetal growth retardation[11], pre-eclampsia[12], and eclampsia[13]. It has been reported that local administration of NOS inhibitor, *L*-NAME to uterine horn of pregnant mice on d 3 led to failure of implantation and retardation of the embryo growth[14]. High NO inhibited both embryo development *in vitro* and implantation *in vivo* in mice[15]. NOS inhibitors show a synergistic effect with antiprogestin on inhibiting the establishment of pregnancy[16]. These findings suggest that the optimal level of nitric oxide is a prerequisite for endometrial receptivity.

Apoptosis in human endometrium has been detected during implantation window period, and that may be important in the processes of decidualization and implantation[17]. The cells undergoing apoptosis have characteristic structural changes in the nucleus and cytoplasm, such as the fragmentation of DNA[18]. These DNA fragments can be detected by terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end-labelling (TUNEL) technique[19,20].

Several proteins such as Fas antigen (Fas)/tumour necrosis factor receptor, Fas ligand (FasL)/TNF, p53, and Myc, in addition to the proteins of the Bcl-2 family, are involved in the regulation of apoptosis. Interaction of Fas and Fas ligand induces apoptosis through autocrine and paracrine signaling[21].

Studies have shown that increased oestrogen later in pregnancy was associated with elevated NOS and cGMP activity[22,23]. On the other hand, oestrogen modulates uterine NO generation[24], therefore, NO may play an intermediary role in the oestrogen-mediated effects on the uterus[25]. Although oestrogen has been reported to be actively involved in inducing endometrial apoptosis to support the process of implantation, the relationship between nitric oxide and apoptosis in endometrium is still unknown. In this study, the effect of abnormal level of NO on the endometrium apoptosis and its potential role of Fas-FasL system in the process of mouse implantation were investigated.

MATERIALS AND METHODS

Animal and reagent Adult female Kunming pregnant mice were purchased from Experimental Animal Institute of Chinese Academy of Medical Sciences (Grade II, Certificate No 01-3001); the restriction enzyme and TdT were purchased from Promega (USA); NBT, BCIP, anti-DIG antibody, blocking reagent, and DIG-ddUTP were purchased from Roche (Germany). Nitric oxide donor molsidomine and nitric oxide synthase inhibitor *N* omega-nitro-*L*-arginine methyl ester (*L*-NAME) were obtained from Sigma (USA). Fas and FasL polyclonal antibody, biotin-conjugated goat anti rabbit IgG and AP-conjugated avidin were purchased from Santa Cruz (USA). Vector-red kit was from Vector (USA); ECL substrate was from PIERCE (USA).

Animal treatment Various amounts of molsidomine (4, 20, and 100 mg/kg) and *L*-NAME (1, 5, and 25 mg/kg) were administered intraperitoneally (ip) into mice in a single dose in 0.9 % saline every 12 h, ten times from 9:00 of d 1 of gestation to d 5 of gestation. Control animals received 0.9 % saline only. Animals were sacrificed by cervical dislocation on d 12 of gestation, and the pregnant dams were calculated. Some of the animals were killed on d 5 of gestation. The uteri were removed and fixed in 4 % formaldehyde solution for 16 h and embedded in paraffin wax for immunohistochemistry and *in situ* hybridization or cryopreseved at -70 ºC for Western blot.

TUNEL *In situ* analysis was performed as described previously[26]. Deparaffinized and hydrated 4 μm sections were subjected to 3'-end-labelling of the DNA with digoxigenindideoxy-UTP (dig-ddUTP; Roche). The standard substrates (nitroblue tetrazolium 337.5 mg/L and 5-bromo-4-chloro-3-inolyl-phosphate 175 mg/L) were used for staining. Negative controls of omission TdT for the technique were included.

***In situ* hybridization** Dig-cRNA probes labeling for Fas and FasL were based on the method of Liu *et al*[27], *in situ* hybridization was carried out based on the method of Schaeren-Wiemers and Gerfin-Moster[28].

Immunohistochemistry Serial 4-μm sections of tissue were cut, deparaffinized, and rehydrated through degraded ethanol. Antigen retrieval was performed by incubating the sections in 0.01 mol/L citrate buffer (pH 6.0) at 98 ºC for 20 min and cooling at room temperature for 20 min. Non-specific binding was blocked with 10 % (v/v) normal goat serum in PBS for 1 h. the sections were incubated with rabbit anti-mouse Fas or rabbit anti-mouse FasL IgG (1 mg/L) in 10 % goat serum for

2 h. The sections were then incubated with biotinylated secondary antibody followed by an avidin-alkaline phosphatase complex and Vector Red substrates, according to the manufacturer's protocol (Vector Laboratories, Burlingame, CA). Endogenous alkaline phosphatase activity was inhibited by supplement of 1 mmol levamisole (Sigma) into Vector Red substrate solution. Rabbit anti-mouse Fas or FasL IgG were replaced with the same concentration of normal rabbit IgG as a negative control. The sections were counterstained with haematoxylin and mounted.

Western blot analysis Western blot was performed as described by Zhou *et al*[29]. Intact mouse uteri were homogenized in lysis buffer (phosphate buffer 5 mmol/L, pH 7.2, containing 0.1 % Triton X-100, phenylmethylsulfonylfluoride 1 mmol/L, leupeptin 1 mg/L, and chymostatin 1 mg/L). The homogenate was clarified by centrifugation, and total protein content of the supernate was determined by spectrophotometer using albumin as the standard. Total cellular protein 50 μg was fractionated in a denaturing 10 % SDS-polyacrylamide gel by electrophoresis. The fractionated samples were then transferred to nitrocellulose membrane, and nonspecific binding was blocked for 1 h in 5 % nonfat dry milk in PBS. The membranes were incubated with anti-Fas antibody for 1 h (1:500). The membranes were washed in PBS, then incubated with HRP-conjugated goat anti-rabbit antibody (1:1000) for 1 h, after washing with PBS, the membranes were incubated with ECL substrate for 5 min. Then the membranes were exposured to X-ray film. The values of fold-increases were determined by densitometric analysis of the stained bands from three independent experiments.

Statistical analysis The quantitative data was expressed mean±SD. Tests from at least 3 individual animals for each treatment group were analyzed. Experiments were repeated at least three times, and one of representatives from at least three similar results was presented. Statistical analysis of pregnancy rates among the groups was performed using chisquare test, $P<0.05$ was considered to be statistically significant.

RESULTS

Effect of abnormal NO levels on pregnancy rate The pregnancy rate in the molsidomine and *L*-NAME treated group (Tab 1) was reduced in a dose-dependent manner.

Tab 1. Effect of molsidomine and *L*-NAME on the pregnancy rate in mice. Chisquare test. [a]$P>0.05$, [b]$P<0.05$, [c]$P<0.01$ *vs* control. *n*= number of animals.

Group	Dose/mg·kg^{-1}	*n*	Pregnancy of animals	Pregnancy rate/%
Control	-	47	46	97.9
Molsidomine	4	18	18	100[a]
	20	19	15	78.9[b]
	100	18	11	61.1[c]
L-NAME	1	18	17	94.4[a]
	5	18	14	77.8[b]
	25	18	8	44.4[c]

Histological changes of endometrium during the implantation window In the normal uterus, the endometrium normally decidualized. After treatment with molsidomine (Fig 1B) and *L*-NAME (Fig 1C), the decidualization was retarded, the character of receptive uterus, as compared with control, the closure of the lumen and the edema of endometrium was markedly reduced. The molsidomine and *L*-NAME treated endometrium showed asynchronized decidualization; the embryos could be found floating in the expanded uterine lumen, but their growth seems not retarded under the current dosage.

TUNEL In order to assess the temporal and spatial distribution of apoptotic cells with DNA strand breaks in endometirum, the experiment of *in situ* 3'-end-labeling of DNA fragment of apoptotic cells was conducted. A large number of apoptotic cells were detected in the endometrium of control group (Fig 2A), but there were only a few positive cells in the molsidomine (Fig 2B) or *L*-NAME (Fig 2C) treated groups.

Immunohistochemistry Staining for Fas in the normal mouse uterus (Fig 3A) was present in the epithelial cells and weaker in the stroma cells; the intensity and the number of positive stroma cells were decreased after molsidomine (Fig 3B) and *L*-NAME (Fig 3C) injection. For FasL, the positive cells were also found in the epithelial cells and the stroma cells of normal and drug treated uteri, but no obvious difference of intensity and the number of positive cells was detected between the *L*-NAME (Fig 3F) treated and the control (Fig 3D) groups; the number of positive stroma cells seemed slightly decreased in the molsidomine treated group (Fig 3E), but no obvious density change was

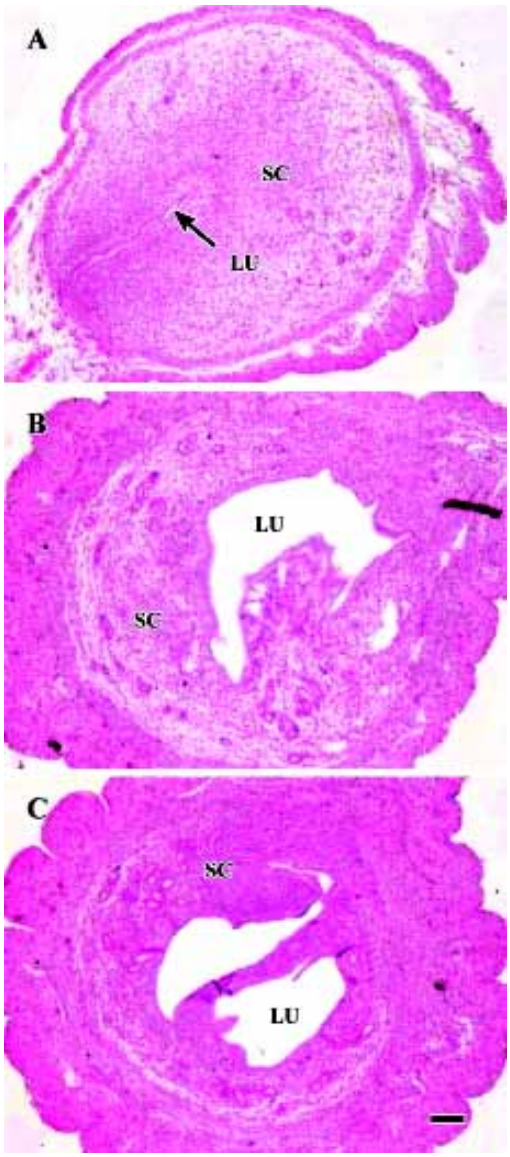

Fig 1. Hematoxylin-eosin staining pregnant uterus on d 5. (A) Normal endometrium. (B) Molsidomine treated, 100 mg/kg. (C) *L*-NAME treated 25 mg/kg. LU, lumen; SC, stroma. Bar=100 μm.

found. No staining was detected in the sections incubated with normal rabbit serum as the negative control (data not shown).

***In situ* hybridization** The expression of Fas and FasL mRNA was assessed by *in situ* hybridization using Fas and FasL antisense dig-cRNA probes in the sections of control, molsidomine and *L*-NAME injection uteri. The expression of Fas mRNA in the control group (Fig 4A) was stronger than that in the molsidomine (Fig 4B) and *L*-NAME (Fig 4C) treated uteri. No obvious FasL change of intensity or number of positive cells was detected between the control (Fig 4D) and the

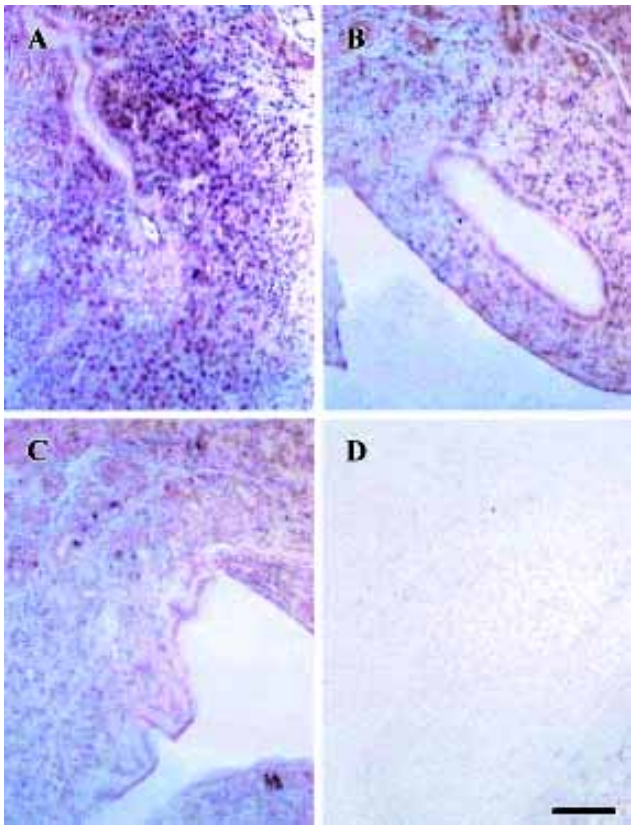

Fig 2. TUNEL analysis for pregnant uterus on d 5. (A) Normal endometrium. (B) Molsidomine treated, 100 mg/kg. (C) *L*-NAME treated, 25 mg/kg. (D) Negative control. Bar=100 μm.

treated (molsidomine, Fig 4E; *L*-NAME, Fig 4F) groups. These findings coincided with the localization of Fas and FasL detected by immunohistochemistry. No staining was found in the sections treated with sense probes of Fas or FasL (data not shown).

Western blot To confirm the expression levels of Fas and FasL protein, Western blotting using the same antibodies as used in immunohistochemistry was carried out. Treatments with molsidomine and *L*-NAME (Fig 5) decreased the expression of Fas (M_r 45000) obviously, but not that of FasL (M_r 31 000).

DISCUSSION

The present study demonstrated for the first time that the obvious apoptosis in the endometrium during implantation period decreased when the normal level of nitric oxide was disturbed, and FasL-Fas pathway may be involved.

The maintenance of the interaction balance among the paracrine molecules such as NO, cytokines, steroid

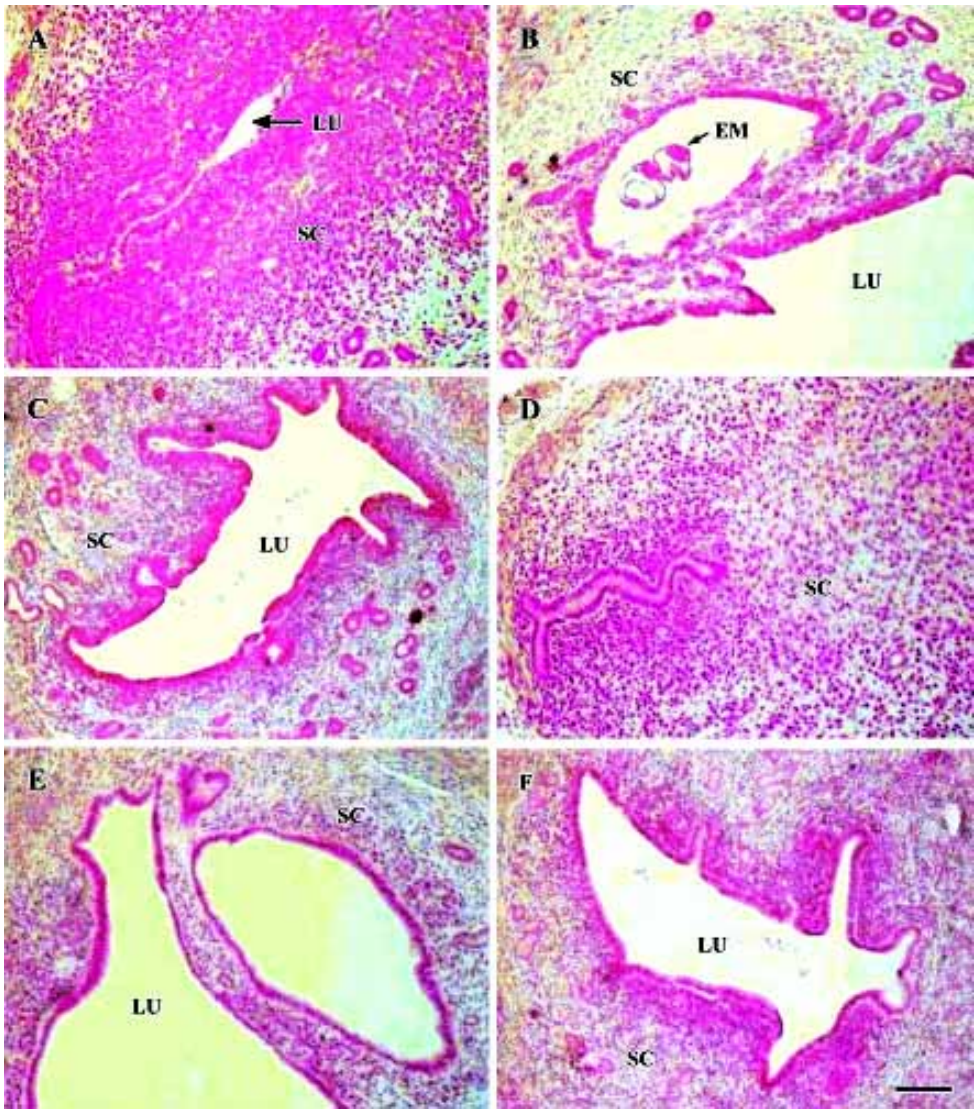

Fig 3. Immunolocalization of Fas (A, B, C) and FasL (D, E, F) on d 5 of gestation. (A, D) Normal endometrium. (B, E) Molsidomine treated, 100 mg/kg. (C, F) *L*-NAME treated, 25 mg/kg. SC, stroma; LU, lumen. Bar=100 μm.

hormones and other molecules may play a significant role in regulation of key events during pregnancy, especially during the implantation period. NO may help establish the proper environment for implantation. The results from this study showed a dose-dependent decrease of pregnant rate in the drug treated groups as compared with the control. This conclusion is consistent with those reported by Ota *et al*[30].

Apoptosis is well recognized as a crucial determinant of successful peri-implantation development, and may be regarded as a marker of uterine receptivity[31-33]. The results of TUNEL in our study showed that there were a large number of apoptotic cells in the normal pregnant endometrium that was consistent with our previous report[34], in which we showed that mifepristone, a widely used prophylactic, partially played its contraceptive role by down-regulating the expression of *Fas* and *FasL*, and suggesting the relationship between the FasL-Fas signaling pathway and the establishment of pregnancy. The result of TUNEL in our study showed that the number of apoptotic cells decreased in the endometrium of the uteri treated with molsidomine or *L*-NAME and therefore linked the decreased apoptosis with the abnormal level of NO. These data tempted us to raise the possibility that the decrease in apoptosis after injection of molsidomine and *L*-NAME might also be regulated by Fas pathway.

As a next step, we examined whether Fas and FasL expression was associated with the change of apoptosis in the endometrium. The results of immunohisto-

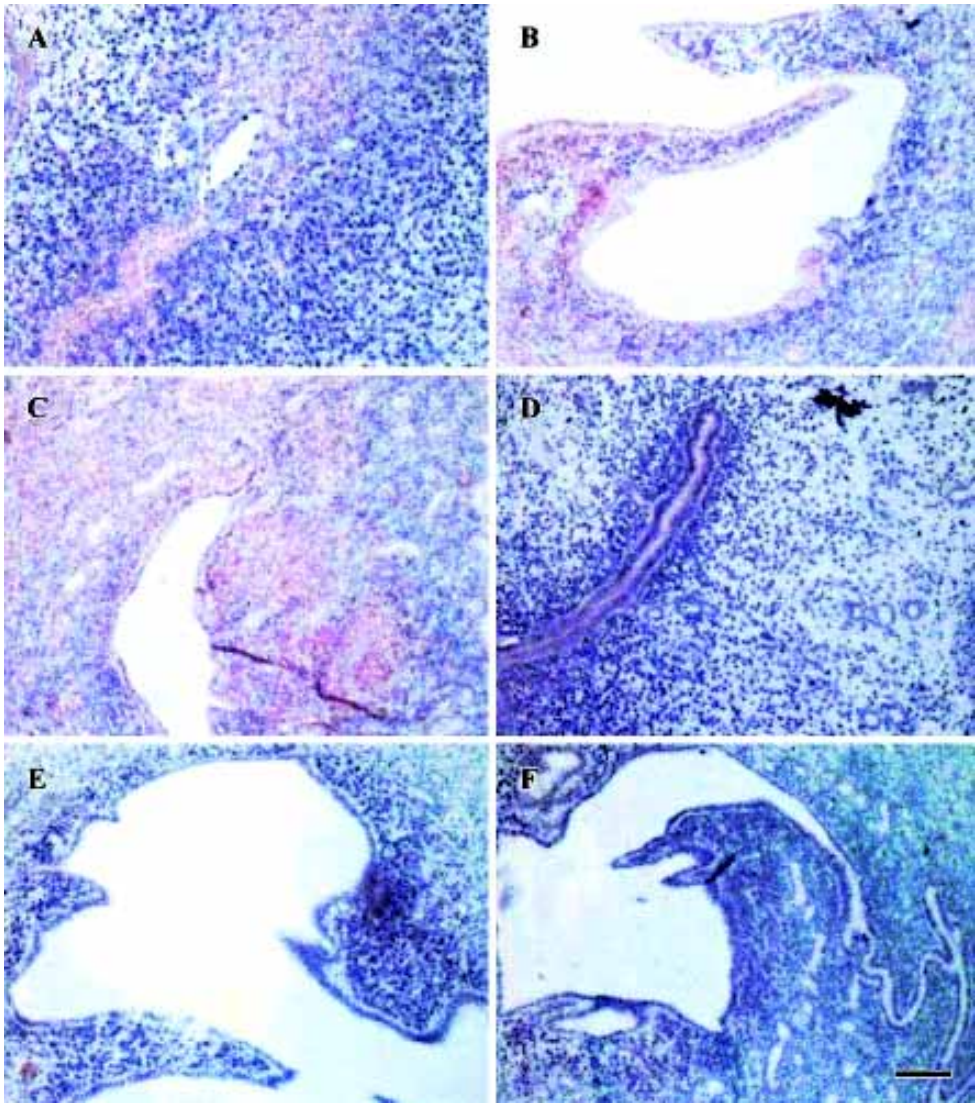

Fig 4. *In situ* hybridization of Fas (A, B, C) and FasL (D, E, F) in the uteri of d 5 gestation. (A, D) Normal endometrium. (B, E) Molsidomine treated, 100 mg/kg. (C, F) *L*-NAME treated, 25 mg/kg. Bar=100 μm

chemistry, *in situ* hybridization and Western blot showed that FasL protein and mRNA changed slightly, whereas Fas proteins and mRNA were relatively down-regulated in the drug treated groups. These results indicated that NO might be one of the regulators in the Fas-mediated apoptosis in the endometrium.

It is well established that the receptivity of endometrium is under the control of oestrogen and progesterone[35,36], and the steroid hormones play an important role in the regulation of endometrial cell apoptosis and NOS expression[37]. On the basis of the results in the present study, we suggested that NO may be one of the regulators involved in the process of hormone-regulated endometrial apoptosis, thus the abnormal NO levels lead to changes in apoptosis which may normally occurred in the receptive endometrium and then decreased receptivity and the failure of embryo implantation. However, *in vitro* studies were needed to further prove this suggestion.

Another possible explanation for the failure of embryo implantation is the developmental arrest of embryo as revealed in oviductal flushing experiments[38] and subcutaneous infusing experiments[39], in which the embryonic development was inhibited beyond the 4-cell stage in mice (treated with NO donor) or around 8-cell stage in rat (treated with NOS inhibitor). But this seems not the main reason in the present study, for blastulas in molsidomine treated uterus (Fig 2B) could still be detected, and the normal implantation still partially happened in molsidomine or *L*-NAME treated uteri.

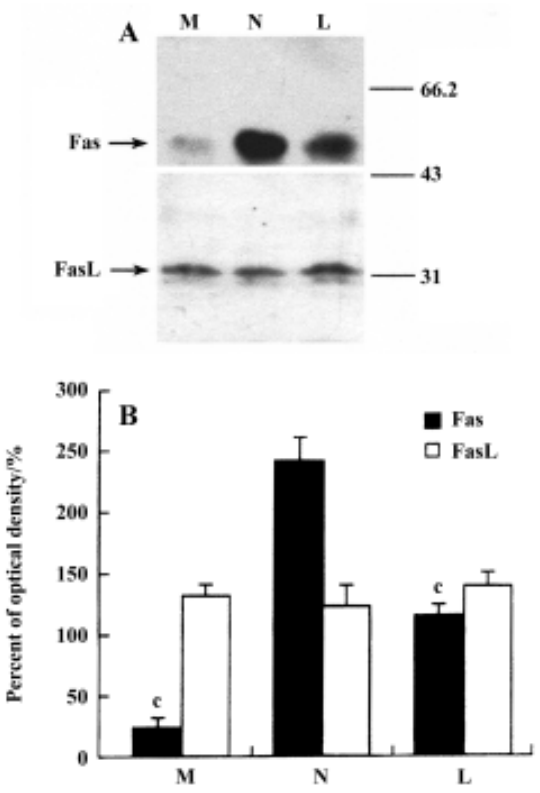

Fig 5. Immunoblot analysis for Fas (M_r 45 000) and FasL (M_r 31 000) antigen. (A) Immublot results of the expression of Fas and FasL proteins. M: molsidomine 100 mg/kg treated. N: normal endometrium. L: *L*-NAME 25 mg/kg treated. (B) Quantitative analysis of Fas and FasL content. n=3. Mean±SD. ^cP<0.01 *vs* normal. (*t*-test).

In conclusion, optimal level of nitric oxide is important for the endometrial receptivity. The changes in nitric oxide level may have affected embryo implantation partially by way of altering the Fas-mediated apoptosis.

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