Role of Nitric Oxide in Ovulation, Meiotic Maturation of Oocytes, and Implantation in Mice

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Nitric oxide synthase blockers inhibited meiotic maturation of oocytes, decreased the count of oocytes during hormonal stimulation of superovulation, and increased embryonic death in experimental females mated with intact males.

Key Words: *nitric oxide; ovulatory productivity; meiotic maturation of oocytes; implantation*

Nitric oxide (NO) is a free radical gas with a half-life in biological objects of about 5 sec. Recent studies demonstrated various functions of this compound. In the vascular system NO induces vasodilation and inhibits platelet aggregation; in the nervous system NO acts as a signal molecule. NO production from L-arginine is catalyzed by cytosolic NO synthase (NOS). There are 2 forms of NOS: constitutive calcium/calmodulin-dependent cNOS (endothelial eNOS and neuronal nNOS) and inducible calcium-independent iNOS. Biochemical and immunohistochemical methods demonstrated the presence of cNOC and iNOS in female reproductive organs [2,8,9]. The oviduct plays an important role in reproduction, regulates transport of gametes and embryos, and maintains specific microenvironment necessary for fertilization and initial stages of embryogenesis. iNOS and eNOS were found in human oviduct [7]. All NOS isoforms are expressed in rat uterine cervix, while the uterus expresses only iNOS and eNOS. During labor, expression of iNOS increases in the uterine cervix, but decreases in the uterus [3]. The role of NO in ovulation, meiotic maturation of oocytes, and implantation is poorly understood. Here we evaluated the role of NO using nonspecific pharmacological NOS blockers N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-monomethyl-L-arginine (L-NMMA) inhibiting all NOS isoforms. The effects of NOS blockers on the count of ovulated oocytes, meiotic maturation of oocytes during hormonal stimulation (HS) of superovulation, and the rate of embryonic death were studied.

MATERIALS AND METHODS

Experiments were carried out on 8-week-old CBA mice weighing 16-20 g (10 females per each dose of NOS blockers and 10 females in each control group).

In series I, female diestrus mice were superovulated. HS of Superovulation was stimulated with equal doses (5 IU) of pregnant mare serum (PMS, Folligon Intervet) and human chorionic gonadotropin (HCG, Profasi, Serono, administered 45-46 h after PMS). Female mice with natural cycle served as the control. Experimental animals received intraperitoneal injections of NOS blockers L-NAME and L-NMMA in single doses of 2.5 and 3.5 mg/kg, respectively, 2 h before and 2 and 6 h after administration of HCG. These doses were selected on the basis of published [5] and experimental data (doses of 0.7, 1.25, 2.5, and 3.5 mg/kg were preliminary tested on CBA females). Control mice received an equivalent volume of physiological saline. Oocytes were obtained from ether-narcotized mice 20 h after HCG injection. The uterine tubes were prepared and transferred into culture medium, and their ampullae were broken. The cells were washed and counted. Meiotic maturation of oocytes

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(metaphase II, normal meiotic stage for ovulated mouse oocytes) was evaluated by the presence of the first polar body (PB). Abnormal meiotic maturation of oocytes was estimated after morphological examination: metaphase I was determined by the presence of germinal vesicles (GV^+). Resumption of the first meiotic division of oocytes was evaluated by the absence of germinal vesicles (GV^-) using an inverted microscope. The content of oocytes with PB and morphologically atypical cells was estimated.

In series II, proestrus females were used. L-NAME and L-NMMA were injected intraperitoneally in single doses of 2.5 and 3.5 mg/kg, respectively. Control mice received an equivalent volume of physiological saline. The barrier separating females and males in a special chamber was removed. Control and treated females were mated with intact males. Mating was confirmed by the presence of vaginal plugs. The indexes of fertility and pregnancy were calculated. The females were killed by cervical dislocation on days 10-12 of pregnancy. We estimated the count of implantation sites in the uterus and number of live and dead embryos. The pre- and postimplantation mortality rates were calculated [1]. The results were analyzed by Student's *t* test.

RESULTS

Injection of NOS blockers to gonadotropin-stimulated mice decreased the mean number of oocytes released through the oviduct (Table 1).

Treatment with L-NAME and L-NMMA before and after administration of HCG reduced the number of metaphase II oocytes compared to the control (Table 2). The decrease in oocyte count was due to inhibition of meiotic and cytoplasmic maturation of oocytes. The inhibition of meiotic maturation of oocytes

TABLE 1. Effects of NOS Blockers on Ovulation and Pre- and Postimplantation Death in CBA Mice ($M \pm m$, n=10)

Parameter	Control	L-NAME	L-NMMA
Mean count of ovulated oocytes	23.0±2.1		
treatment with blockers			
2 h after HCG injection		15.0±3.0**	16.5±2.0
6 h after HCG injection		12.8±2.3*	13.5±2.0*
Preimplantation mortality, %	2.7±1.6	8.2±2.3**	4.3±1.7
Postimplantation mortality, %	1.3±0.8	7.1±0.8*	4.4±0.7

Note. *p<0.01 and **p<0.05 compared to the control.

TABLE 2. Effect of NOS Blockers on Oocyte Maturation

Parameter	Treatment with NOS blockers		
	2 h before HS	2 h after HS	6 h after HS
Number of metaphase II oocytes, % of the control			
L-NAME	84.0	88.5	87.0
L-NMMA	80.0	87.0	84.0
Number of arrested oocytes, %			
GV^+			
L-NAME	10	12	4
L-NMMA	4	—	—
GV^-			
L-NAME	4	6	4
L-NMMA	6	8	12
Count of cells with enlarged perivitelline space, fragmented cytoplasm, *etc.*, %*			
L-NAME	2	2	3.5
L-NMMA	3	5	4

Note. *Not found in the control.

manifested in enlargement of the perivitelline space, appearance of irregular granularity in the cytoplasm and fragmentation of the cytoplasm (Table 2). These changes were not detected in control animals.

Thus, NOS blockers decrease the count of superovulated oocytes. This indicates that ovarian NOS is essential for maximal ovulation, while the absence of NO in the preovulatory period leads to serious disturbances in mitotic maturation of oocytes. Injection of NOS blockers 6 h after HCG administration most significantly decreased the ovulatory productivity in female mice.

NOS blockers did not change the fertility index in female mice. After treatment with NOS blockers the index of pregnancy did not differ from the control. However, 20-40% matings were ineffective over the first 10 days after administration of L-NAME or L-NMMA, hence NOS blockers inhibited early pregnancy. It should be emphasized that females fertilized over the first 4 days after treatment were nonpregnant. The pre- and postimplantation rates of embryonic mortality increased in females mated with intact males in the early stage after treatment with test preparations (Table 1).

Thus, NOS blockers increase the embryonic mortality rate in mice. This can be related to abnormal meiotic maturation of oocytes, which causes zygote death and impairment of the mother-fetus relationships.

Our results are consistent with published data on the role of eNOS-produced NO in ovarian steroidogenesis and ovulation in outbred female mice and animals carrying mutant eNOS gene [4], changes in NOS activity in the ovaries of immature rats during ovulation [5], and iNOS content in mouse uterine tissues on days 4-8 of pregnancy [6].

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