Arch Pharm Res Vol 27, No 1, 86-93, 2004

Archives of Pharmacal Research
http://apr.psk.or.kr

Involvement of Nitric Oxide During *In Vitro* Fertilization and Early Embryonic Development in Mice

Bo Hyun Kim, Chang Hong Kim, Kyu Young Jung[1], Byung Hun Jeon[2], Eun Jin Ju[3], and Young Kug Choo[3]

Eden Obstetric & Gynecology Clinic, Iksan, Jeonbuk 570-982, Korea, [1]Department of Pharmacology, Wonkwang University School of Medicine, Iksan, Jeonbuk 570-749, Korea, [2]Department of Pathology, College of Oriental Medicine, Wonkwang University, Iksan, Jeonbuk 570-749, Korea, and [3]Department of Biological science, College of Natural Sciences and Medicinal Resources Research Center, Wonkwang University, Iksan, Jeonbuk 570-749, Korea

(Received October 2, 2003)

Nitric oxide (NO) has emerged as an important intracellular and intercellular messenger, controlling many physiological processes and participating in the fertilization process *via* the autocrine and paracrine mechanisms. This study investigated whether nitric oxide synthase (NOS) inhibitior (L-NAME) and L-arginine could regulate *in vitro* fertilization and early embryonic development in mice. Mouse epididymal spermatozoa, oocytes, and embryos were incubated in mediums of variable conditions with and without L-NAME or L-arginine (0.5, 1, 5 and 10 mM). Fertilization rate and early embryonic development were significantly inhibited by treating sperms or oocytes with L-NAME (93. 8% vs 66.3%, 92.1% *vs* 60.3%), but not with L-arginine. In contrast, fertilization rate and early embryonic development were conspicuously reduced when L-NAME or L-arginine was added to the culture media for embryos. Early embryonic development was inhibited by microinjection of L-NAME into the fertilized embryos in a dose-dependent manner, but only by high concentrations of L-arginine. These results suggest that a moderate amount of NO production is essential for fertilization and early embryo development in mice.

Key words: Early embryonic development, *In vitro* fertilization, Nitric oxide, L-NAME, L-Arginine

INTRODUCTION

A free radical molecule, nitric oxide (NO), is a double-edged sword, serving as a key signal molecule in both physiological and pathological processes such as vasodilation (Ignarro *et al.*, 1987), neurotransmission (Bredit *et al.*, 1989; Garthwaite *et al.*, 1988; Izumi *et al.*, 1992), and macrophage cytotoxicity (Hibbs *et al.*, 1987). Because neurons, blood vessels, and the immune system are integral parts of the reproductive organs, it is likely that NO is an important regulator of the reproductive system. The limited data concerning the involvement of NO in sperm fertilizing ability suggest that a low concentration of NO results in a significant increase in sperm capacitation, although there are conflicting reports concerning the effects of NO on sperm motility and viability (Hellstrom *et al.*, 1994; Nobunaga *et al.*, 1996; Rosselli *et al.*, 1995; Weinberg *et al.*, 1995; Zini *et al.*, 1995). Reports have shown that NO can be generated by spermatozoa. Moreover, immunoreactivity for cNOS was observed in mouse (Herrero *et al.*, 1996a) and human sperm (Herrero *et al.*, 1996b; Lewis *et al.*, 1996; O`Beyan *et al.*, 1998). In mouse sperm, a NOS isoform was revealed at Western blot analysis as a unique band of M_r = 140 KDa, recognized by both eNOS and bNOS antibodies (Herrero *et al.*, 1997). However, the definitive effect of NO on sperm zona pellucida binding and the acrosome reaction, which are prerequisites to successful fertilization, has not been fully determined.

It has been suggested that L-NAME, the nonspecific nitric oxide synthase (NOS) inhibitor, arrests embryo development (Gouge *et al.*, 1998); administration of NOS inhibitor in rat uteri during the implantation period, thus, interferes with implantation as it retards embryo development and inhibits the endometrial bed (Biswas *et al.*, 1998). NO is a well recognized activator of guanylate cyclase that induces an increase in cyclic guanosine monophosphate (cGMP) levels in target cells (Moncada

Correspondence to: Young-Kug Choo, Ph.D, Department of Biological Science, College of Natural Sciences, Wonkwang University, 344-2 Shinyoung-dong, Iksan, Jeonbuk 570-749, Korea
Tel: 82-63-850-6087, Fax: 82-63-857-8837
E-mail: ykchoo@wonkwang.ac.kr

et al., 1991; Nathan., 1992). The importance of the ratio of cyclic adenosine monophosphate (cAMP) to cGMP in the regulation of preimplantation embryonic development and differentiation has been previously proposed (Dey *et al.*, 1978). While we were carrying out this study, Chen *et al.* (2001) reported an interesting data showing the importance of NO in the early stages of mouse embryonic development. However, the precise mechanism of NO in the embryonic development still remains unclear. Using a specific inhibitor and precursor of NO, this study examined whether NO could regulate the rates of fertilization and early embryonic development in mice in order to demonstrate the essential role of NO in the regulation of fertilization and early embryonic development.

MATERIALS AND METHODS

Culture media and reagents

The media used in *in vitro* fertilization (IVF) of oocytes were Toyoda, Yokoyama, and Hosi (TYH: 119.4 mM Nacl, 4.8 mM KCl, 1.7 mM $CaCl_2{\cdot}2H_2O$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4{\cdot}7H_2O$, 25.1 mM $NaHCO_3$, 5.5 mM glucose, 1 mM Na-pyruvate, 0.2 mM penicillin G, 0.034 mM streptomycin and 320 mg/80 mL bovine serum albumin (BSA)), and Modified Whitten's medium (MWM: 109.5 mM NaCl, 3.2 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4.7H_2O$, 22.6 mM $NaHCO_3$, 1.5 mM calcium lactate-5-hydrate, 5.5 mM glucose, 0.22 mM Na-pyruvate, 0.034 mM streptomycin, 0.2 mM penicillin G, 20 mM β mercaptoethanol, 100 mM ethylenediaminetetraacetic acid (EDTA) and 240 mg/80 mL BSA)). The culture medium used for early development of mouse embryos was MWM. All fertilization and culture media were covered with paraffin oil (Fisher, USA) and equilibrated overnight in an atmosphere of 5% CO_2 at 37°C. L-NAME and L-arginine were directly dissolved in TYH or MWM media and used immediately.

Animals and superovulation

Hybrid F1 (C3H/N×C57 BL/6N) was used in all experiments. Animals were housed under controlled conditions of 14 h light and 10 h darkness at a temperature of 23± 2°C and relative humidity of 55-66%. To induce superovulation, female mice were treated with 5 IU of pregnant mare's serum gonadotropin (PMSG, Sigma, St Louis, USA), followed by injection of human chorionic gonadotropin (hCG, 5 IU) 48 h after PMSG treatment. Mice were sacrificed by cervical dislocation 15 h after hCG administration, and both oviducts were immediately removed and placed in TYH medium.

Preparation of sperm suspension

According to standard procedures (Choi *et al.*, 2002; Jung *et al.*, 2001; Kwak *et al.*, 2003; Oh *et al.*, 1998; Toyoda *et al.*, 1974), a dense mass of spermatozoa was isolated from the epididymis of 10-week old B6C3F1 mice and placed in 200 μL of TYH, which had been covered with paraffin oil, and then incubated at 37°C in 5% CO_2 atmosphere for 1 h. Sperm concentrations were determined with a haemocytometer.

Oocyte collection

Oocytes were collected from oviducts 14 h after administration of hCG for superovulation. The oviducts were isolated and placed in a dish containing paraffin oil. The cumulus-oocyte complexes were dissected from swollen ampulla and transferred to TYH medium under paraffin oil, followed by preincubation at 37°C in 5% CO_2.

In vitro fertilization

In vitro fertilization was carried out in drops of TYH under paraffin oil. A capacitated sperm suspension was gently added to the freshly ovulated oocytes to give a final motile sperm concentration of 1×10^6/mL. The combined sperm-oocyte suspension was incubated for 5 h. The oocytes were then washed through several media and finally incubated in 40 drops of MWM medium under paraffin oil in the presence or absence of drugs, L-NAME, and L-arginine. Fertilization was confirmed by recording the number of 2-cell embryos at 24 h after completion of *in vitro* fertilization.

Micromanipulation

Fertilized embryos were incubated in TYH medium for 15-30 min, and 50 μL of the oocyte-containing medium was transferred to the glass plate. Using glass micropipettes, MWM medium containing L-NAME or L-arginine was injected into the cytoplasm of fertilized embryos. For the control experiments, MWM medium was injected into the cytoplasm of fertilized embryos. The injection volume was approximately 5 μL. After manipulation, the embryos were cultured in individual microdrops (about 50 μL) under light mineral oil.

Statistical analysis

Each experiment replicated at least five times. Data were presented as mean±SD. Student's *t* test was used to evaluate statistical significance of differences between paired observations. A value of $p<0.05$ was considered statistically significant.

RESULTS

Effects of L-NAME and L-arginine on sperm

Firstly, this study examined whether L-NAME or L-

arginine treatment (sperm only or both sperm and media) could affect the fertilization rate and early embryonic development; the results obtained are shown in Table I. After treating the sperm with or without each drug, *in vitro* fertilization was carried out, followed by incubation of the fertilized embryos in the absence of each drug (Table IA). Percentages of morulars and blastocysts were reduced in the group treated with 5 mM L-NAME (92.1% *vs* 84.5%, 92.1% vs 83.4%). Treatment of sperm with L-NAME (10 mM) significantly inhibited the fertilization rate and embryonic development ($p<0.05$), but 0.5, 1, and 5 mM L-arginine had no significant effect on *in vitro* fertilization rate and embryonic development. Ten mM L-arginine inhibited the percentage of embryonic development (92.1% vs 84.5%).

After treating the sperm with or without each drug, *in vitro* fertilization was carried out, followed by incubation of the fertilized embryos in the presence of each drug (Table IB). L-NAME (5 and 10 mM) significantly reduced fertilization and early embryonic development, but L-arginine did not affect them.

Effects of L-NAME and L-arginine on oocyte

This study examined whether L-NAME or L-arginine treatment (oocyte only or both oocyte and media) could affect the fertilization rate and early embryonic development; the results obtained are shown in Table II. After the oocytes were treated with or without each drug, *in vitro* fertilization was carried out, followed by incubation of the fertilized embryos in the absence of each drug (Table IIA). Treatment of oocytes with L-NAME (5 and 10 mM) significantly inhibited the fertilization rate (89.5% vs 77.1%, 89.5% vs 71.3%) and embryonic development (85.5 % *vs* 65.9%, 85.5% vs 52.9%), but L-arginine (0.5,1 and 10 mM) had no significant effect. Five mM L-arginine increased embryonic development (85% vs 91.9%).

After the oocytes were treated with or without each drug, *in vitro* fertilization was carried out, followed by incubation of the fertilized embryos in the presence of each drug (Table IIB). All concentrations of the L-NAME used significantly inhibited fertilization and early embryonic development. However, only high concentrations (10 mM)

Table I. Effects of L-NAME and L-arginine on sperm fertilization rate and embryonic development

A

	Concentration	No. of oocytes	No. of Fertilized oocytes (%)	No. of 2-cell (%)	No. of 4-cell (%)	No. of Morula (%)	No. of Blastocyst (%)
Control		35	93.8±0.83	93.8±0.83	93.8±0.83	92.1±2.51	92.1±2.51
L-NAME	0.5 mM	30	90.1±4.09	88.9±4.61	88.9±4.61	87.7±2.70	87.7±2.70
	1 mM	33	91.3±6.13	90.4±6.56	90.4±6.56	85.1±8.11	84.3±6.49
	5 mM	32	93.0±2.14	89.7±6.81	88.4±5.91	82.7±7.57*	82.7±7.57*
	10 mM	32	66.3±6.00*	65.0±6.77*	65.0±6.77*	60.3±7.21*	60.3±7.21*
L-arginine	0.5 mM	37	96.7±3.86	96.7±3.86	91.7±8.40	84.8±6.38	82.3±9.89
	1 mM	35	95.6±3.41	92.4±7.04	91.2±5.52	84.1±7.88	84.1±7.88
	5 mM	30	90.4±3.10	90.4±3.10	88.9±5.59	87.5±4.31	87.5±4.31
	10 mM	31	90.4±4.11	90.4±4.11	87.3±7.35	84.5±6.32*	83.4±6.47*

B

	Concentration	No. of oocytes	No. of Fertilized oocytes (%)	No. of 2-cell (%)	No. of 4-cell (%)	No. of Morula (%)	No. of Blastocyst (%)
Control		32	92.2±3.13	92.2±3.13	90.5±3.97	85.8±4.26	85.8±4.26
L-NAME	0.5 mM	30	92.9±2.80	88.0±6.59	88.0±6.59	76.2±7.51	76.2±7.51
	1 mM	33	90.0±4.69	84.7±11.2	83.7±9.88	52.5±5.00*	52.5±5.00*
	5 mM	35	87.1±3.45	78.6±2.40*	00*	00*	00*
	10 mM	31	69.0±7.52*	66.5±11.90*	00*	00*	00*
L-arginine	0.5 mM	33	89.8±3.86	88.4±5.12	88.4±5.12	86.3±2.77	86.3±2.77
	1 mM	30	91.5±3.31	89.8±3.72	89.8±3.72	86.8±4.86	86.8±4.86
	5 mM	32	91.8±3.90	88.8±4.37	87.7±6.43	85.6±5.86	85.6±5.86
	10 mM	34	91.7±2.76	90.8±4.25	90.8±4.25	89.9±5.94	89.9±5.94

Before *in vitro* fertilization, sperms were treated with or without each drug, and fertilized oocytes were incubated in the absence (A) or prese nce (B) of each drug. Each value represents the mean±SE. *$p<0.05$ *vs* control.

Table II. Effects of L-NAME and L-arginine on oocyte fertilization rate and embryonic development

A

	Concentration	No. of oocytes	No. of Fertilized oocytes (%)	No. of 2-cell (%)	No. of 4-cell (%)	No. of Morula (%)	No. of Blastocyst (%)
Control		33	89.5±5.08	85.0± 3.56	85.0± 3.56	82.7± 5.48	81.4±4.81
L-NAME	0.5 mM	30	91.7±4.91	89.4± 4.65	87.1± 4.42	85.8± 3.60	83.6±2.07
	1 mM	34	87.6±5.19	84.4± 6.73	83.0±40.1	80.6± 6.24	79.1±8.47
	5 mM	35	77.1±7.69*	72.5± 4.44*	71.2± 5.84*	69.6± 5.98*	65.9±9.64*
	10 mM	37	71.3±7.27*	65.4±11.79*	63.1± 7.75*	55.1±11.76*	52.9±7.24*
L-arginine	0.5 mM	32	90.0±5.13	85.9± 8.81	85.9± 8.81	85.9± 8.81	85.9±8.81
	1 mM	35	92.2±2.16	90.9± 1.69	89.7± 3.49	88.6± 3.68	88.6±3.68
	5 mM	30	93.0±5.03*	91.9± 4.70*	91.9± 4.70*	91.9± 4.70*	91.9±4.70
	10 mM	34	91.0±9.37	89.8± 8.91	89.8± 8.91	89.8± 8.91	89.8±8.91

B

	Concentration	No. of oocytes	No. of Fertilized oocytes (%)	No. of 2-cell (%)	No. of 4-cell (%)	No. of Morula (%)	No. of Blastocyst (%)
Control		36	92.2±1.63	91.3±2.04	91.3±2.04	88.5±4.88	88.5±4.88
L-NAME	0.5 mM	34	83.0±0.79*	81.3±2.12*	81.3±2.12*	63.1±4.78*	63.1±4.78*
	1 mM	30	76.5±1.30*	70.0±6.81*	70.0±6.81*	55.5±7.62*	55.5±7.61*
	5 mM	37	74.1±5.11*	68.5±6.55*	00*	00*	00*
	10 mM	33	68.9±4.61*	62.8±3.07*	00*	00*	00*
L-arginine	0.5 mM	35	91.9±4.75	88.0±6.25	88.0±6.25	83.4±8.56	83.4±8.56
	1 mM	32	92.5±5.07	90.2±7.00	90.2±7.00	85.8±7.53	85.8±7.53
	5 mM	33	88.2±5.28	85.9±5.10	85.9±5.10	82.4±4.61	80.8±5.81
	10 mM	30	88.4±4.87	86.7±2.93*	84.9±4.42*	83.4±3.96	83.4±3.96

Before *in vitro* fertilization, oocytes were treated with or without each drug, and fertilized oocytes were incubated in the absence (A) or pres ence (B) of each drug. Each value represents the mean±SE. *$p<0.05$ vs control.

of L-arginine significantly disturbed the early stages of embryonic development.

Effects of L-NAME and L-arginine on sperm and oocyte

This study examined whether L-NAME- or L-arginine treatment (sperm *plus* oocyte only or both sperm *plus* oocyte and media) could affect fertilization rate and early embryonic development; the results obtained are shown in Table III. After the sperm and oocytes were treated with or without each drug, *in vitro* fertilization was carried out, followed by incubation of the fertilized embryos in the absence of each drug (Table IIIA). Treatment of sperm and oocyte with L-NAME (1 to 10 mM) significantly inhibited the fertilization rate and embryonic development ($p<0.05$), but L-arginine had no significant effect on *in vitro* fertilization rate and embryonic development.

After the sperm and oocytes were treated with or without each drug, *in vitro* fertilization was carried out, followed by incubation of the fertilized embryos in the presence of each drug (Table IIIB). All concentrations of the L-NAME tested significantly inhibited *in vitro* fertilization and early embryonic development. Interestingly, L-arginine showed a biphasic effect on the *in vitro* fertilization and embryonic development. Low concentrations (0.5 mM) of L-arginine stimulated the percentage of *in vitro* fertilization (91.5% vs 96.4%) and embryonic development (84.7% vs 92.1%), but the difference was not statistically significant. However, high concentrations (10 mM) did conspicuously inhibit fertilization and embryonic development.

Microinjection of L-NAME and L-arginine into fertilized embryos

Effects of L-NAME and L-arginine on the embryonic development were investigated by direct microinjection of each drug into the fertilized embryo cytoplasm. As shown in Table IV, L-NAME dramatically inhibited embryonic development in a dose-dependent manner ($p<0.05$). In contrast, low concentrations of L-arginine stimulated the embryo development.

Table III. Effects of L-NAME and L-arginine on sperm and oocyte fertilization rate and embryonic development

A

	Concentration	No. of oocytes	No. of Fertilized oocytes (%)	No. of 2-cell (%)	No. of 4-cell (%)	No. of Morula (%)	No. of Blastocyst (%)
Control		30	89.9±5.08	89.9±5.08	89.9±5.08	85.0±6.79	85.0±6.79
L-NAME	0.5 mM	34	87.3±5.94	83.8±7.56	82.2±5.95	78.8±8.58	77.7±7.80
	1 mM	32	79.8±2.55*	76.7±3.88*	76.7±3.88*	73.2±6.32*	72.7±6.86*
	5 mM	37	81.2±4.01*	76.9±9.41*	75.1±7.72*	68.9±8.07*	68.9±8.07*
	10 mM	33	74.0±5.03*	69.5±5.94*	69.5±5.94*	65.2±6.63*	62.7±7.66*
L-arginine	0.5 mM	32	90.4±2.57	89.6±1.38	89.6±1.38	86.3±2.11	83.0±4.34
	1 mM	35	90.3±3.83	88.6±6.46	88.6±6.46	85.8±8.39	84.7±7.68
	5 mM	33	93.9±1.20	89.5±3.33	89.5±3.33	87.6±4.17	87.6±4.17
	10 mM	37	93.9±3.11	91.7±4.12	89.5±6.16	88.9±5.13	88.9±5.13

B

	Concentration	No. of oocytes	No. of Fertilized oocytes (%)	No. of 2-cell (%)	No. of 4-cell (%)	No. of Morula (%)	No. of Blastocyst (%)
Control		30	91.5± 3.45	88.9± 5.09	88.9± 5.09	85.3±6.96	84.7±6.44
L-NAME	0.5 mM	32	82.6± 1.41*	79.0± 3.46*	79.0± 3.46*	63.4±9.73*	62.1±7.60*
	1 mM	34	75.0± 1.88*	71.2± 6.20*	64.9±11.44*	60.0±8.24*	56.8±5.20*
	5 mM	37	74.1± 4.18*	71.0± 6.82*	00*	00*	00*
	10 mM	35	63.4±11.87*	61.3±11.51*	00*	00*	00*
L-arginine	0.5 mM	37	96.4± 1.30	93.2± 3.51	93.2± 3.51	92.1±4.75	92.1±4.75
	1 mM	30	93.0± 2.14	89.3± 4.37	89.3± 4.37	85.5±5.89	85.5±5.89
	5 mM	34	89.1± 1.40	85.5± 3.91	85.5± 3.91	83.7±6.67	83.7±6.67
	10 mM	32	80.5± 1.76*	75.0± 3.15*	71.8± 3.68*	66.6±1.58*	66.6±1.58*

Before *in vitro* fertilization, the sperm and oocytes were treated with or without each drug, and fertilized oocytes were incubated in the absen ce (A) or presence (B) of each drug. Each value represents the mean±SE. *$p<0.05$ vs control.

Table IV. Microinjection of L-NAME and L-arginine in fertilized oocytes cytoplasm

	Concentration	No. of oocytes	No. of 2-cell (%)	No. of 4-cell (%)	No. of Morula (%)	No. of Blastocyst (%)
Control		32	63.9±4.28	63.9±4.28	61.6±6.40	61.6±6.40
L-NAME	0.5 mM	30	53.6±1.68*	53.6±1.68*	50.1±4.87*	50.1±4.87*
	1 mM	32	45.4±5.32*	45.4±5.32*	41.3±3.99*	41.3±3.99*
	5 mM	33	40.3±5.80*	40.3±5.80*	35.2±8.40*	35.2±8.40*
	10 mM	28	25.2±8.80*	25.2±8.80*	19.8±7.13*	19.8±7.13*
L-arginine	0.5 mM	29	75.7±3.08*	75.7±3.08*	72.4±3.17*	72.4±3.17*
	1 mM	30	70.8±3.56	69.1±5.26	67.9±3.77	65.6±4.50
	5 mM	33	66.9±1.56	64.4±3.17	62.0±5.59	60.4±4.80
	10 mM	31	60.9±4.23	60.9±4.23	57.1±5.56	57.1±5.56

L-NAME and L-arginine into cytoplasm of fertilized oocytes were microinjected. Oocytes with two pronuclei 5 h after insemination were scored as fertilized. Ooytes were collected from oviducts 14-15 h after hCG injection. Microinjections were completed with 20 h after hCG injection. Each value represents the mean±SE. *$p<0.05$ *vs* control.

DISCUSSION

This study demonstrated that L-NAME, the NOS inhibitor, inhibited the fertilization rate and embryonic development in a dose-dependent manner and that low concentrations of L-arginine, the NO donor, stimulated the fertilization rate and embryonic development, which, however, were inhibited by high concentrations of L-arginine. These results suggest

that a moderated amount of NO production was essential for fertilization and embryo development and that excessive amounts of NO generation inhibit fertilization and embryo development.

The discovery of the key role of NO in several biological functions has recently sparked a developing area of research into the reproductive system. NO is synthesized in the adrenal and pituitary glands and testis, where NOS blockage has been shown to affect testosterone production (Adams *et al.*, 1992). Because NOS has also been localized in the epididymis (Burnett *et al.*, 1995) and has demonstrated a diverse spectrum of activities, hypothetically it may have a significant role on sperm function.

Sperm capacitation - the molecular basis of which remains largely unknown - is associated with changes of membrane lipid composition, ion channel activation, intracellular second-messenger production, and protein tyrosine phosphorylation (Baldi *et al.*, 1996). The present study attempted to evaluate the effects of NO on the fertilization rate and early embryonic development and observed that sperm incubated with L-NAME exhibited significantly reduced fertilization rate and early embryonic development, whereas 0.5, 1, and 5 mM L-arginine had no affect (Table IA and IB). L-NAME on the fertilization has been suggested to suppress capacitation and/or interfere with the dynamics of acrosomal exocytosis. These results lead us to consider that blockage of NO production in sperm may lead to the inhibition of *in vitro* fertilization and early embryonic development. This suggestion is also supported by previous reports from Gouge *et al.* (1998), Herrero *et al.* (1996a), Revelli *et al.* (1999) and Yeoman *et al.* (1998), which suggested that NO affects fertilization and embryonic development.

Several studies have indicated that NO binds to and activates soluble guanylyl cyclase and increases cGMP levels in target cells (Schmidt *et al.*, 1993). It has been suggested that cGMP lowers the cAMP level by activating oocyte cAMP-phosphodiesterase (cAMP-PDE) and thus permits oocyte maturation to continue (Hubbard and Price, 1988). When mouse oocytes were treated with L-NAME or L-arginine, *in vitro* fertilization rate and early embryonic development were significantly reduced by L-NAME, but not by L-arginine (Table IIA and IIB). It is likely that the physiological level of NO is involved in the process of oocytes maturation and fertilization. These results are well consistent with the previous results reported by Kuo *et al.* (2000), which demonstrated that NOS and nitric oxide-related bioactivity satisfy the primary criteria of an egg activator: they were present in an appropriate place, active at an appropriate time and sufficient for successful fertilization in sea urchin. Considering these previous reports and our results, the NO pathway in mouse oocytes is thought to be of essential importance in preimplantation embryo development. However, further studies are required to evaluate the exact influential mechanisms of NO/NOS system in mouse oocytes implicated in *in vitro* fertilization and early embryonic development.

Researchers have previously demonstrated that administration of NOS inhibitor inhibits embryo development in mice (Barroso *et al.*, 1998). Gagioti *et al.* (2000) reported that murine blastocyst and post-implantation trophoblast cells positively strained both eNOS and iNOS protein and produced high levels of NO. We demonstrated that addition of NOS inhibitor at relatively high concentrations (5, 10 mM) inhibits development of preimplantation for mouse embryo, especially the conversion of the 2-cell embryo into the 4-cell (Table IB, IIB and IIIB). These observations suggest varying levels of NO may have different regulatory effects on developing embryos at different stages.

On the other hand, treatment of sperms and/or oocytes with relatively low concentrations of L-arginine conspicuously promoted *in vitro* fertilization and embryo development, but not high concentrations of L-arginine (Table IIIB). Based on these results and previous reports (Barroso *et al.*, 1998), we consider that appropriate amounts of NO may be essential in maintaining normal embryo development. However, the molecular mechanism for the NO-regulated embryo development remains to be clarified. Previous studies have shown that NO synthesis increases during pregnancy, but decreases at the end of gestation (Sladex *et al.*, 1997; 1993). The microinjection of L-NAME into fertilized embryo cytoplasm decreased the number of 2-cell embryos developing to the 4-cell, morula, and blastocyst stages, but the microinjection of 0.5 mM L-arginine into fertilized embryo cytoplasm increased early embryonic development (Table IV). These results indicate that NO may be a key regulator of signal transduction during early embryonic development.

We conclude that L-NAME significantly inhibits the percentage of early embryonic development in a dose-dependent manner. However, low concentrations of L-arginine increase early embryonic development. These findings suggest that NO may play a crucial role in fertilization and embryogenesis. The observed difference in required amount of NO and the cytotoxicity of NO in each developmental stage may also suggest that the NO/NOS system is tightly regulated in a developmental stage -specific manner.

Abbreviations with four tables abbreviations

PMSG : pregnant mares serum gonadotropin
hCG : human chorionic gonadotropin
L-NAME : N^G-nitro-L-arginine methyl ester

ACKNOWLEDGMENTS

We thank Dr. M. Hoshi (Graduate School of Science and

Technology, Keio University) for numerous suggestions and comments on the subjects. This work was supported by the Wonkwang University in 2003.

REFERENCES

Adams, M. L., Nock, B., Trung, R., and Cicero, T. J., Nitric oxide control of steroidogenesis: endocrine effects of N^G-nitro-L-arginine and comparisons to alcohol. *Life. Sci.,* 50, PL35-40 (1992).

Barroso, R. P., Osuamkpe, C., Nagamani, M., and Yallampalli, C., Nitric oxide inhibits development of embryos and implantation in mice. *Mol. Hum. Reprod.*, 4, 503-507 (1998).

Biswas, S., Kabir, S., and Pal, A. K., The role of nitric oxide in the process of implantation in rats. *J. Reprod. Fertil.,* 114, 157-161 (1998).

Baldi, E., Luconi, M., Bonaccorsi, L., Krausz, C., and Forti, G., Human sperm activation during capacitation and acrosome reaction: role of calcium, protein phosphorylation and lipid remodelling pathways. *Front. Biosci.,* 1, d189-205 (1996).

Burnett, A. L., Ricker, D. D., Chamness, S. L., Maguire, M. P., Crone, J. K., Bredt, D. S., Snyder, S. H., and Chang, T. S., Localization of nitric oxide synthase in the reproductive organs of the male rat. *Biol. Reprod.,* 52, 1-7 (1995).

Bredt, D. S. and Synder, S. H., Nitric oxide mediates gultamate-linked enhancement of cGMP levels in the cerebellum. *Proc. Natl. Acad. USA,* 86, 9030-9033 (1989).

Choi, H. G., Kim, J. K., Cho, J. R., Kim, B. J., Jung, K. Y., Choi, B. K., Shin, M. K., and Choo. Y. K., Effects of high molecular weight water-soluble chitosan on in vitro fertilization and ovulation in mice fed a high-fat diet. *Arch. Pharm. Res.,* 25, 178-183 (2002).

Chen, H. W., Jiang, W. S., and Tzeng, C. R., Nitric oxide as a regulator in preimplantation embryo development and apoptosis. *Fertil. Steril.*, 75, 1163-1171 (2001).

Dey, S. K., Kimura, F., Mukherjee, F., and Dickmann, Z. J., Cyclic-AMP and cyclic-GMP in rabbit blastocysts. *Reprod. Fertil.,* 52, 235-237 (1978).

Gagiot, S., Scavone, C., and Bevilacqua, E., Participation of the mouse implanting trophoblast in nitric oxide production during pregnancy. *Biol. Reprod.,* 62, 260-268 (2000).

Gouge, R. C., Marshburn, P., Gordon, B. E., Nunley, W., and Huet-Hudson, Y. M., Nitric oxide as a regulator of embryonic development. *Biol. Reprod.,* 58, 875-879 (1998).

Garthwait, J., Charles, J, S., and Chess-Williams, R., Endothelium-derived relaxing factor release on activation of NMDA receptors suggests roles as intracellular messenger in the brain. *Nature. London,* 336, 385-388 (1988).

Herrero, M. B., Goin, J. C., Boquet, M., Canteros, M. G., Franchi, A. M., Perez Martinez, S., Polak, J. M., Viggiano, J. M., and de Gimeno, M. A. F., The nitric oxide synthase of mouse spermatozoa. *FEBS. Lett.*, 411, 39-42 (1997).

Herrero, M. B., Viggiano, J. M., Perez Martinez, S., and de Gimeno, M. A. F., Effects of nitric oxide synthase inhibitors on the outcome of *in vitro* fertilization in the mouse. *Reprod. Fertil. Dev.*, 8, 301-314 (1996a).

Herrero, M. B., Perez Martinez, S., Viggiano, J. M., Polak, J. M., and de Gimeno, M. F., Localization by indirect immuno-fluorescence of nitric oxide synthase in mouse and human spermatozoa. *Reprod. Fertil. Dev.*, 8, 931-934 (1996b).

Hellstrom, W. J. G., Bell, M., Wang. R., and Sikka, S. C., Effect of sodium nitroprusside on sperm motility, viability, and lipid peroxidation. *Fertil. Steril.,* 61, 1117-1122 (1994).

Hubbard, C. J. and Price, J., The effects of follicle-stimulating hormone and cyclic guanosine 3', 5'-monophosphate and cyclic adenosine 3', 5'-monophosphate-phosphodiesterase and resumption of meiosis in hamster cumulus-oocyte complexes. *Biol. Reprod.,* 39, 829-838 (1988).

Hibbs, J. B., Taintor, Jr. R. R., and Vavrin, Z., Macrophage cytotoxicity: role for L-arginine deminase and imino nitrogen oxidation to nitrite. *Science. Wash. DC.,* 325, 473-476 (1987).

Izumi, Y., Clifford, D. B., and Zorumski, C. F., Inhibition of long-term potentiation by NMDA-mediated nitric oxide release. *Science. Wash. DC.,* 257, 1273-1276 (1992).

Ignarro, L. J., Buga, G. M., Wood, K. S., Byrns, R. E., and Chaudhuri, G., Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. USA,* 84, 9265-9269 (1987).

Jung, K. Y., Kim, B. H., Hwang, M. R., Cho, J. R., Kim, H. M., Lee, Y. C., Kim, C. H., Kim, J. K., and Choo, Y. K., Differential distribution of ganglioside GM3 in seminiferous tubule and epididymis of adult rats. *Arch. Pharm. Res.,* 24, 360-366 (2001).

Kwak, D. H., Jung, K. Y., Lee, Y. C., and Choo, Y. K., Expressional changes of ganglioside GM3 during ovarian maturation and early development in db/db mice. *Develop. Growth. Differ.,* 45, 95-102 (2003).

Kuo, R. C., Baxter, G. T., Thompson, S. H., Stricker, S. A., Patton, C., Bonaventura, J., and Epel, D., NO is necessary and sufficient for egg activation at fertilization. *Nature,* 406, 633-636 (2000).

Lewis, S. E. M., Donnelly, E. T., Sterling, E. S. L., Kennedy, M. S., Thompson, W., and Chakravarthy, U., Nitric oxide synthase and nitrite production in human spermatozoa: evidence that endogenous nitric oxide is beneficial to sperm motility. *Mol. Hum. Reprod.*, 2, 873-878 (1996).

Moncada, S., Palmer, R. M. J., and Higgs, E. A., Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmcol. Rev.,* 43, 109-142 (1991).

Nobunaga, T., Takugawa, Y., Hashimoto, K., Kubota, Y., Sawai, K., and Kimura, T., Elevated nitric oxide concentration in the seminal plasma of infertile males: nitric oxide inhibits sperm motility. *Am. J. Rep. Immunol.*, 36, 197-197 (1996).

Nathan, C., Nitric oxide as a secretory product of mammalian cells. *FASEB J.*, 6, 3051-3064 (1992).

Oh, S. H., Miyoshi, K., and Funahashi, H., Rat oocytes fertilized

in modified rat 1-cell embryo culture medium containing a high sodium chloride concentration and bovine serum albumin maintain developmental ability to the blastocyst stage. *Biol. Reprod.,* 59, 884-889 (1998).

O'Bryan, M. K., Zini, A., Cheng, G. Y., and Schlegel, P. N., Human sperm endothelial nitric oxide synthase expression: correlation with sperm motility. *Fertil. Steril.*, 70, 1143-1147 (1998).

Revelli, A., Soldati, G., Costamagna, C., Pellerey, O., Aldieri, E., Massobrio, M., Bosia, A., and Ghigo, D., Follicular fluid proteins stimulate nitric oxide (NO) synthase in human sperm: a possible role for NO in acrosomal reaction. *J. Cell Physiol.*, 178, 85-92 (1999).

Rosselli, M., Dubey, R. K., Imthurn, B., Macas, E., and Keller, P., Effects of nitric oxide on human spermatozoa: evidence that nitric oxide decrease sperm motility and induces sperm toxicity. *Hum. Reprod.*, 10, 1786-1790 (1995).

Sladex, S. M., Magness, R. R., and Conrad, K. P., Nitric oxide and pregnancy. *Am. J. Physiol.,* 272, R441-R463 (1997).

Sladek, S. M., Regenstein, A. C., Lykins, D., and Roberts, J. M., Nitric oxide synthase activity in pregnant rabbit uterus decreases on the last day of pregnancy. *Am. J. Obstet. Gynecol.,* 160, 1285-1291 (1993).

Schmidt, H. H. H. W., Lohmann, S. M., and Walter, U., The nitric oxide and cGMP signal transduction system: regulation and mechanism of action. *Biochim. Biophys. Acta,* 1178, 153-175 (1993).

Toyoda, Y. and Chang, M. C., Fertilization of rat eggs *in vitro* by epididymal spermatozoa and the development of eggs following transfer. *J. Reprod. Fertil.*, 36, 9-22 (1974).

Weinberg, J. B., Doty, E., Bonaventura, J., and Haney, A. F., Nitric oxide inhibition of human sperm motility. *Fertil. Steril.,* 62, 1244-1249 (1995).

Yeoman, R. R., Jones, W. D., and Rizk, B. M., Evidence for nitric oxide regulation of hamster sperm hyperactivation. *J. Androl.,* 1, 58-64 (1998).

Zini, A., Lamirande, E. D., and Gagnon, C., Low levels of nitric oxide promote human sperm capacitation *in vitro. J. Androl.,* 16, 424-431 (1995).