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Nitric oxide as a regulator in preimplantation embryo development and apoptosis

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Objective: To investigate the mechanisms of nitric oxide (NO) in the development and apoptosis of preimplantation mouse embryos.

Design: Prospective, controlled study.

Setting: Medical college laboratory.

Subject(s): Two-cell embryos from outbred ICR mice.

Intervention(s): Hyperstimulation protocol, two-cell embryos were collected, then treated with or without an NO synthase inhibitor (L-NAME) or an NO donor (SNP) and combined with a cGMP analogue (8-Br-cGMP) or a selective inhibitor of NO-sensitive soluble guanylyl cyclase (ODQ).

Main Outcome Measure(s): The development of ICR mouse embryo from two cells to blastocyst stages in vitro.

Result(s): The development of blastocyst was inhibited by L-NAME in a concentration-dependent manner (0.1–10 μM) and 0.1 μM SNP reversed this effect (80.5% of control). Annexin-V/propidium iodide and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling techniques demonstrated that excessive NO ($\geq$10 μM) might induce apoptosis in the mouse embryos. 8-Br-cGMP reversed the inhibitory effect of L-NAME and rescued the embryo growth. ODQ inhibited the embryo development in a dose-responsive fashion (0.1–100 μM) but had no effect in the NO-induced embryo apoptosis. P53 and Bax were found to be up-regulated during the embryo fragmentation.

Conclusion(s): These results indicate that the cGMP pathway might be involved in the NO-regulated embryonic development, but not in NO-induced apoptosis, for which P53/Bax pathway might be involved. (Fertil Steril® 2001;75:1163–71. ©2001 by American Society for Reproductive Medicine.)

Key Words: Apoptosis, c-GMP, embryo development, nitric oxide, p53

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Nitric oxide (NO), a free radical molecule, is a double-edged sword, serving as a key-signal molecule in both physiological and pathological process (1–3). NO that is generated by neurons can act as a neurotransmitter; it can be generated by macrophages in response to invading microbes, when it acts as an antimicrobial agent (4, 5). Within the vasculature, NO is involved in induction of vasodilation, inhibition of platelet aggregation, smooth muscle cell proliferation and migration, regulation of apoptosis, and maintenance of endothelial cell barrier function (6–8). Because neurons, blood vessels, and the immune system are integral parts of the reproductive organs, consequently, it is likely that NO is an important regulator of the reproductive system. There is evidence supporting NO involvement in steroidogenesis, hormone-regulated ovulation, and atresia-related apoptosis (9–11).

NO is produced when NO synthase (NOS) catalyzes the oxidation of L-arginine to L-citrullin. Various isoforms of NOS have been isolated, including endothelial NOS (eNOS); inflammatory, macrophage, and inducible NOS (iNOS); and neuronal NOS (nNOS or bNOS; see References 1–3). These three isoforms of NOS are reported to express in the uterus and cervix and have been suggested as being important for embryo implantation, pregnancy, and labor (9, 12–15). N^G-nitro-L-arginine, the nonspecific NOS inhibitor, has been reported to arrest embryo development (16). Gouge et al. previously published data showing the impor-

tance of both iNOS and eNOS in early stages of mouse embryonic development (16). However, the precise mechanisms of NO in the embryonic development remain unknown, especially from a molecular point of view.

Ota et al. found that both iNOS and eNOS expression increased during endometriosis and adenomyosis and suggested a correlation with early embryo loss (17). The NO molecule controls programmed cell death (apoptosis) by up-regulation of the tumor suppressor p53, then by changes in the expression of pro- and antiapoptotic Bcl-2 family members, cytochrome C relocation, activation of caspases, chromatin condensation, and DNA fragmentation (18). Diethylenetriamin/NO, an NO donor, has been reported to inhibit the embryo development and implantation in mice (19). Lim and Hansel reported that blastocyst formation of bovine oocytes cocultured with granulosa cells was significantly increased after addition of hemoglobin (Hb, 1 mg/mL), an NO scavenger (20). These studies indicate that NO might be an embryotoxic substance.

The aims of this study were to investigate the mechanisms of NO in the preimplantation embryonic development and to find out whether the NO-induced embryo degeneration was apoptotic. It has been previously proposed that cGMP may be important in the regulation of preimplantation embryonic growth and differentiation (16, 21–23). Because cGMP mediates many effects of NO (24), this study was conducted to examine the hypothesis that NO affects preimplantation embryo development through the cGMP pathway.

Additionally, large amounts of NO can also promote breakage of DNA strands, which can initiate apoptosis and cause cell death in various cell types (25–28). A previous study reported that the role of macrophage activation in early embryo loss was determined by the release of NO (29). NO-induced embryonic apoptosis in this study was also examined. The apoptosis of embryos induced by excess NO might be the reason why inflammatory diseases such as endometriosis induce early embryo loss and lead to lower pregnancy rates.

MATERIALS AND METHODS

Animals

This study was approved by the Institutional Review Board and the Animal Care and Use Committee of the Taipei Medical College. The animal experiments reported in this study were performed in adherence to the guidelines established in the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Health Research Institutes (NHRI). All animals used were humanely cared for. Outbred strains of ICR mice (random bred) (Harlan Sprague-Dawley, Indianapolis, IN) were kept on a 10-hour light, 14-hour dark cycle and allowed free access to drinking water and laboratory chow.

Female mice at 6 weeks of age were superovulated by i.p. injection of 5 IU of pregnant mare serum gonadotropin (PMSG; Sigma Chemical Co., St. Louis, MO), followed (48 h) by an i.p. injection of 10 IU of hCG (Sigma). They were then housed overnight with virile males. The following morning, females were checked for the presence of a vaginal plug. Previously mated mice were used for this study.

Embryo Culture and Development Study

After mating, female mice were killed on day 2 by cervical dislocation, and the oviducts were flushed with Dulbecco's phosphate-buffered saline (PBS; Gibco, Grand Island, NY) with a 1-mL syringe and pipette to collect two- to four-cell embryos under a dissecting microscope. Twenty to thirty embryos were usually obtained from each animal, two-cell embryos were collected and cultured in human tubal fluid (HTF; Santa Ana, CA) containing 0.3% of bovine serum albumin (BSA; Sigma; see References 30, 31).

The embryos were randomly distributed, with about 100 embryos used in each treatment group of the study. The embryos were incubated at 37°C under 90% N_2, 5% CO_2, and 5% O_2 and were examined under the microscope at 24-hour intervals.

All embryos were classified one of the following: as degenerated (fragmented and lysis), two-cell, four-cell, 8- to 16-cell, compacted (morula), early blastocyst, late blastocyst, and hatched (16, 19). The media containing N^G-nitro-L-arginine methyl ester (L-NAME, NOS inhibitor; Sigma), sodium nitroprusside (SNP, a NO donor; Sigma), 8-bromoguanosine 3′,5′-cyclic monophosphate (Br-cGMP, an analogue of cGMP that can pass through the cell membrane; Sigma), and/or 1H-(1,2,4) oxadiaxlol-(4,3-a) quinoxalin-1-one (ODQ, selective inhibitor of nitric oxide–sensitive soluble guanylyl cyclase; Sigma) were freshly prepared throughout the study.

Apoptosis Detection

Propidium iodide (PI) and annexin V staining of living fragmented embryos were performed as described by Van Blerkom and Davis to determine cell membrane integrity and presence of phosphatidylserine residues on the outer surface of the plasma membrane, respectively (32). Annexin V is a protein that binds to phosphatidylserine residues, which are exposed on the surface of apoptotic cells but not on normal cells. In healthy cells, the distribution of the phosphatidylserine groups in the plasma membrane is asymmetrical with the phosphatidylserine groups being directed toward the interior of the cell. However, during apoptosis, this asymmetry is lost, and the phosphatidylserines are exposed to the exterior of the cell. Annexin V staining is therefore an established biochemical marker of apoptosis.

The partial loss of membrane integrity or functionality is a useful criterion for distinguishing apoptotic from necrotic and living cells. PI is a standard probe that is used to distinguish viable cells from nonviable cells. Viable cells or early apoptotic cells with more intact membranes exclude PI,

whereas membranes of necrotic cells are permeable to PI. Cells that stain positive for annexin V–FITC and negative for PI are in the early stages of apoptosis. Cells that stain positive for both annexin V–FITC and PI are either in the later stages of apoptosis, are undergoing necrosis, or are already dead. Cells that are negative for both annexin V–FITC and PI staining are viable and not undergoing measurable apoptosis (32). Embryos were first incubated at 37°C for 45 minutes in PBS containing PI (500 ng/mL) and then examined by fluorescence microscopy after incubation with a FITC conjugate of annexin V (1 mg/mL).

Detection of apoptotic nuclei was accomplished by in situ nuclear labeling with terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) reaction (Boehringer Mannheim, Laval, Quebec, Canada), as described elsewhere (33, 34). Zona-free embryos at the different stages were fixed in 3.7% paraformaldehyde solution in PBS for 30 minutes, rinsed twice in PBS, and permeated with 0.1% Triton-X 100 in 0.1% sodium citrate for 2 minutes on ice. Embryos were resuspended in 50 μL of TUNEL reaction mixture or 50 μL of TUNEL label alone as a negative control. Embryos were incubated for 60 minutes at 37°C in a humidified atmosphere. Positive controls were incubated with 50 U/mL of RNase-free DNase (Promega, Madison, WI) for 20 minutes at 37°C before exposure to the TUNEL reaction mixture. Embryos were rinsed twice in PBS and examined with the fluorescent microscope.

Immunocytochemistry

The expression of iNOS, eNOS, p53, and Bax protein products in embryos were examined by immunocytochemistry with specific antibody (16). Embryos were fixed in 3.7% paraformaldehyde solution in PBS for 30 minutes, rinsed twice in PBS, and permeated with 0.1% Triton X-100/PBS for 10 minutes. Embryos were blocked with PBS containing 3% goat serum for 30 minutes and then incubated for 4 hours at room temperature with 1:200 dilution of the primary antibody of iNOS, eNOS, p53 (Transduction Laboratories, Lexington, KY), or Bax (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in a humidified chamber.

After washes with PBS, the embryos were incubated for 1 hour with the secondary antibody, anti-rabbit IgG or anti-mouse IgG, FITC conjugated for iNOS and eNOS, and HRP (peroxidase) conjugated for p53 and Bax (Santa Cruz Biotechnology Inc., Santa Cruz, CA). They then were examined with the fluorescent microscope (iNOS and eNOS) or incubated with the ABC mixture (Vector Laboratories, Inc., Burlingame, CA; p53 and Bax) for 1.5 hours. The embryos were washed again with PBS, and the antigen–antibody complexes were visualized by immersion in a 3,3′-diaminobenzidine (DAB) solution (Vector Laboratories, Inc.). The embryos were then counterstained with methyl green, dehydrated in graded ethanol baths and xylene, and mounted with a coverslip for light microscopy. Negative controls were processed in the same manner but were not incubated with the primary antibody. All antibodies had been previously characterized through immunocytochemistry, and Western blot analysis was specific for iNOS, eNOS, p53, and Bax (Transduction Lab, Technical notes; unpublished data).

Statistical Analysis

Data are presented as mean ± SEM. Paired Student's *t* test was used to evaluate statistical significance of differences between paired observations. A value of $P<.05$ was considered statistically significant.

RESULTS

NO Production Is Essential for Mouse Embryo Development

In the study, the eNOS and iNOS immunostaining in the two-cell, four-cell, morula, and blastocyst stages were examined in ICR mice embryos, but was more significant in immunostaining only at blastocyst stage (Fig. 1B, C), compared with the negative control group (Fig. 1A). The in vitro development of mouse embryos was monitored daily from day 1 to day 4 during culture in HTF medium with or without different concentrations of NOS inhibitor (L-NAME). In the control media, 76.9 ± 4.1% (73/95) of the embryos developed to blastocyst stage at day 3, and 47.9 ± 1.2% (45/95) of embryos developed to hatching stage at day 4 (Fig. 2). L-NAME (0.1–10 μM) inhibited the embryo development to blastocyst and hatching stages in a concentration-dependent manner. However, no significant difference in the percentage of embryonic development within six- to eight-cell stage was observed when L-NAME was used at the concentrations of 0.1 to 10 μM ($P>.05$; Fig. 2).

In a further study for cytotoxicity of L-NAME, embryos were cultured for 24 hours in medium with 10 μM L-NAME, then washed twice with PBS and then incubated in the fresh medium without L-NAME. After another 24 hours, there was no difference in development to blastocyst stage compared with the control groups (Table 1).

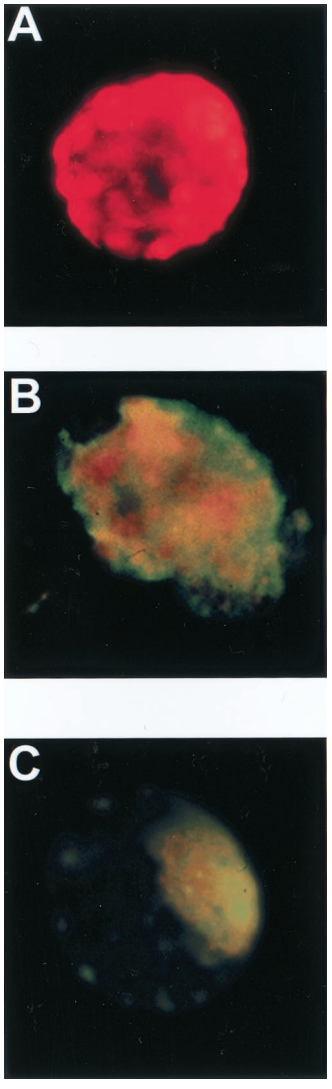
To investigate the direct evidence whether the NO is essential for embryo development, the NO donor, SNP, was directly added to the medium containing L-NAME. The results show that the inhibitory effect of L-NAME (10 μM) on embryo development could be rescued by 0.1-μM SNP (62.4% blastocyst on day 3 and 27.1% hatching on day 4, $P<.05$; Table 1).

cGMP Involved in the NO-Regulated Embryo Development

To investigate the signal transduction pathway of NO in the preimplantion embryo development, the involvement cGMP pathway in the NO essential development of embryo was evaluated. The cGMP donor, 8-Br-cGMP (1 μM), was added to the medium containing 10-μM L-NAME. The direct evidence was that the inhibitory effect of L-NAME on embryo development could be partially rescued by the ana-

FIGURE 1

Immunocytochemical staining of eNOS and iNOS on embryos by day 3 of incubation. The fixed zona-free embryos were stained with the following FITC-conjugated polyclonal antibody: (**A**) Negative control and (**B**) eNOS and (**C**) iNOS. ×400.



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logue of cGMP (1 μM 8-Br-cGMP, 62.0% blastocyst on day 3 and 29.4% hatching on day 4; Table 1). A selective soluble guanylate cyclase inhibitor, ODQ, was used to further determine the role of cGMP pathways in mediating development of embryos induced by NO. Our results indicated that ODQ (0.1–100 μM) inhibited embryo development in a concentration-dependent manner (Table 2).

NO-Induced Embryo Apoptosis

The embryos cultured with SNP were assessed to determine whether they made a successful transition from one developmental stage to the next. Development of day 1 embryos was growth from the two-cell stage to the four-cell stage or beyond, then to eight-cell stage on day 2, to morula on day 3, and blastocyst on day 4. In control embryos, 91.3% developed to the next stage on day 1, 71.6% on day 2, 76.9% on day 3, and 47.9% developed to the blastocyst stage on day 4. In the embryos treated with 0.1-μM SNP, there were 91.1% of embryos developed to the next stage on day 1, 72.1% on day 2, 77.1% on day 3, and 50.5% of the embryos developed to the blastocyst stage on day 4.

These results showed that there were no significant differences in the embryo development treated with 0.1 μM SNP as compared with the control group. However, none of the embryos progressed beyond the blastocyst stage (0 of 24) when SNP was used at concentration of 10 μM ($P<.05$). The NO donor, SNP, therefore appeared to inhibit embryo development from two-cell to blastocyst stages at 10 μM.

When the embryos treated with SNP, the embryos were degenerated in a time- and concentration-dependent manner (Fig. 3). Four days after the incubation with 1.0-μM and 10.0-μM SNP, degenerated embryo (arrest, fragmentation, and lysis) was observed (55.5% and 87.5%), respectively, but not at the level of 0.1-μM SNP (1.5%, $P<.05$, Fig. 3).

The annexin V procedure stains cell membrane that has lost the membrane integrity, a characteristic of cells in the early stages of apoptotic cell death. Using annexin-V (FITC conjugated, green fluorescence) and PI (red fluorescence for chromatin staining) staining, 77.3% of degenerated embryonic membrane was annexin-V positive (strong green fluorescence, Fig. 4B), the control group only showed the background green fluorescence, Fig. 4A). Some of the embryos showed the double staining with green and red, indicating that the embryo was necrotic (21.3%).

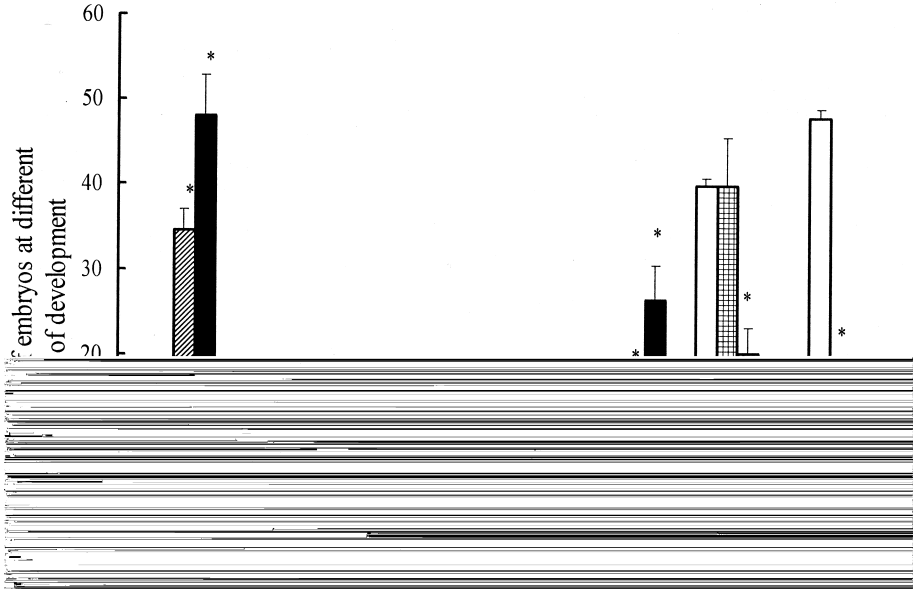
The TUNEL procedure stains nuclei that contain nicked DNA, a characteristic exhibited by cells in the early stages of apoptosis, and the normally developed embryos were negative for TUNEL staining (only positive for PI staining; Fig. 4C). In contrast, a large number of specific TUNEL-positive nuclei were detected in embryos after treatment with 10-μM SNP (Fig. 4D, 30.0%, 15/50). In the TUNEL-positive (green-fluorescence) embryos, the stain localized in the cell nuclei rather than in their cytoplasm was shown in some of the fragmented embryo (Fig. 4D).

Up-Regulated p53 and Bax in NO-Induced Embryo Apoptosis

NO-induced embryo apoptosis was not inhibited by coincubation with ODQ (0.1–10 μM; data not shown) and high concentration of 8-Br-cGMP (10 μM) also cannot induce embryo apoptosis alone (2.8% apoptosis on day 4 with 10 μM 8-Br-cGMP), indicating that this effect might be cGMP independent. Immunocytochemical staining indicated that 10-μM SNP–induced fragmentation of embryo had higher expression of p53 or Bax protein. Most of the p53 or Bax-

FIGURE 2

In vitro preimplantation development of mouse embryos on day 4 after culture in medium without treatment (□, control) or in media supplemented with different concentrations of L-NAME (NOS inhibitor; ▦ = 0.1 μM; ▨ = 1.0 μM; ■ = 10.0 μM). Stage of development for each embryo was assessed daily, and the percentages of embryos at different developmental stages within each group are presented. Total number of embryos per treatment group was 80–100. Values are mean ± SE. *Significantly different from control value, $P<.05$ by paired Student's *t*-test.



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positive (brown-colored) cells were fragmented in the embryos (Fig. 5B, D). The normal development embryo only expressed p53 in the inner cell mass (Fig. 5A), but no Bax expression was observed (Fig. 5C). The cells had no p53 or Bax expressed were visualized by counterstaining with methyl green (p53 or Bax-negative, green colored). Negative controls were processed in the same manner but were not incubated with the primary antibody (data not shown).

DISCUSSION

This study provides the first reported evidence for involvement of cGMP in the signal transduction pathway of NO-regulated embryo development. It thus demonstrates that apoptosis induced by nonphysiological levels of NO is cGMP independent, and it identifies an up-regulated p53 expression conductive to programmed cell death, indicating

TABLE 1

In vitro cultured mouse embryos in the medium containing different compound treatments, showing the percentage of embryonic development that had been attained at blastocyst stage, hatching, and degeneration by day 3 (D3) and day 4 (D4) of incubation.

Treatment	Blastocyst (%) (D3)	Hatching (%) (D4)	Degeneration (%) (D4)
Control	77 ± 4 (73/95)	48 ± 1 (45/95)	8 ± 3 (6/95)
L-NAME (10 μM)	4 ± 2 (2/59)[a]	0 (0/59)[a]	48 ± 5 (29/59)[a]
L-NAME (10 μM) + washout (at D2)	71 ± 4 (41/58)[b]	57 ± 3 (33/58)[b]	9 ± 1 (5/58)[b]
L-NAME (10μM) + SNP (0.1 μM)	62 ± 3 (59/98)[b]	27 ± 2 (24/98)[b]	35 ± 3 (27/98)[b]
L-NAME (10 μM) + 8-Br-cGMP (1 μM)	68 ± 9 (41/63)[b]	33 ± 11 (19/63)[b]	15 ± 3 (10/63)[b]

Note: Values represent the mean ± SE.

[a] $P<0.05$ when compared with the control group.

[b] $P<0.05$ when compared with the L-NAME (10 μM)–treated group, paired Student's *t*-test.

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TABLE 2

Concentration-dependent response of selective inhibitor of nitric oxide–sensitive soluble quanylyl cyclase (ODQ) on mouse embryo development.

Group	Concentration (μM)	Blastocyst (%) (D3)	Hatching (D4)
Control	—	77 ± 4 (73/95)	48 ± 1 (45/95)
ODQ	0.1	67 ± 11 (64/96)	39 ± 3 (38/96)[a]
	1.0	46 ± 7 (40/86)[a]	30 ± 4 (23/86)[a]
	10	8 ± 2 (6/80)[a]	6 ± 1 (4/80)[a]
	100	0 (0/88)[a]	0 (0/88)[a]

Note: Values are mean ± SE.

[a] Significantly different from control value, $P<0.05$, paired Student's *t*-test.

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the possibility of the involvement of independent molecular pathways in NO-induced apoptosis. Mouse embryos at the blastocyst stage were stained positively with both eNOS and iNOS primary antibodies, confirming the previous data in CD-1 mice (16). These results indicate that NO production might be essential for the development of preimplantation embryos.

The NOS inhibitor, L-NAME, concentration dependently inhibited the growth of the mouse embryos; and the NO donor, SNP, arrested embryo growth and induced embryo apoptosis at high dosage. These results suggest that a moderate amount of NO production was essential for embryo development, but that excessive amount of NO generation might inhibit embryo development and thus lead to infertility.

FIGURE 3

In vitro preimplantation development of mouse embryos in medium without treatment (□, control) and in media supplemented with different concentrations of SNP (▦ = 0.1 μM; ▨ = 1.0 μM; ■ = 10.0 μM). The degeneration (fragment, arrest, and lysis) for each embryo was assessed daily, and the percentage of degenerated embryos within each group are presented. Total number of embryos per treatment group was 80–100. Values are mean ± SE. *Significantly different from control value, $P<.05$ by paired Student's *t* test.

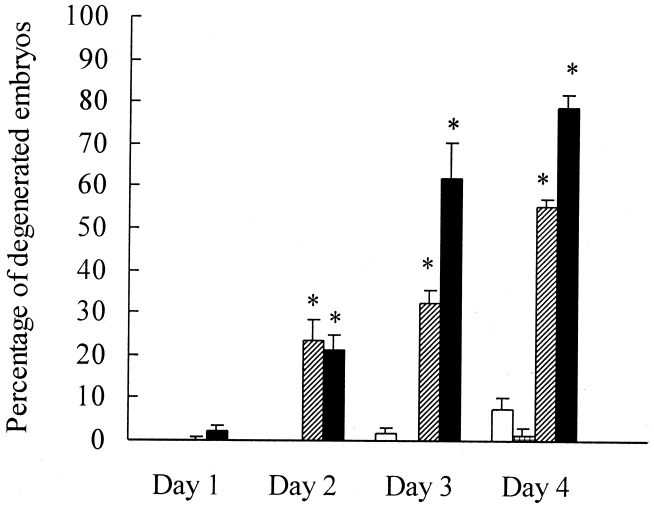

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FIGURE 4

In vitro preimplantation development of mouse embryos in medium without treatment (control, panels **A** and **C**) or in media supplemented with 10-μM SNP (NO donor, panels **B** and **D**) were collected for the study of apoptosis. These representative embryos were examined by annexin-V (green fluorescence; panels **A** and **B**) or TUNEL reaction (green fluorescence), and propidium iodide (red fluorescence) counterstained (panels **C** and **D**), ×200. The normal embryo showed no staining with annexin-V (**A**) or TUNEL (**C**), but the fragmented embryo had strong green-fluorescence (**B**; annexin V positive) or yellow fluorescence (**D**, TUNEL positive, double stained with TUNEL and propidium iodide). In the annexin V–positive cells, the stain localized in the cell membrane, and the staining localization of TUNEL-positive cells was in the cell nuclei rather than in their cytoplasm.

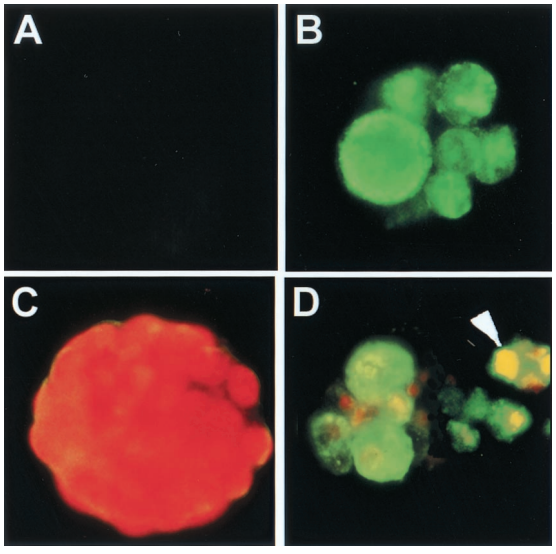

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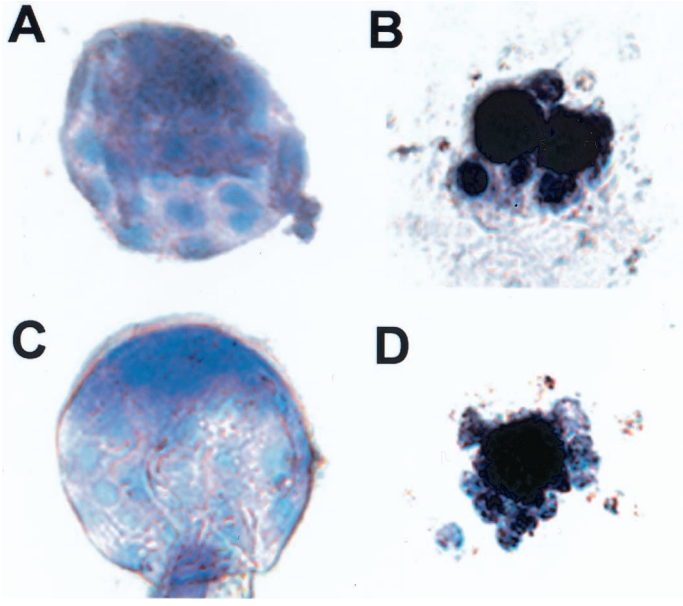
FIGURE 5

Immunocytochemical localization of p53 (**A** and **B**) and Bax (**C** and **D**) on embryos after SNP treatment is shown as follows: control embryo (**A** and **C**) and fragmented embryo (10-μM SNP treatment for 4 days, panels **B** and **D**); $\times$200. The brown-colored cells (or fragments) indicated the p53- or Bax-positive staining, and the negative cells were visualized by counterstaining with methyl green (green colored).



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According to these results, the NO/cGMP pathway is thought to be of essential importance in preimplantation embryo development. There are three reasons to support this point of view. First of all, cyclic nucleotides (cAMP and cGMP) were suggested to play a role on cell mitosis, differentiation, and transformation in the blastocyst or in some cell type (35, 36). This suggests that the inhibition of NO alters cGMP production, which is detrimental to normal embryonic development.

Second, the first differentiation event occurring in the preimplantation mouse blastocyst is the formation of inner cell mass–derived primitive endoderm cells, some of which migrate over the inner surface of the trophectoderm to establish the parietal endoderm layer (37). This evidence indicated that the motility and migration of cells played a major role in the early embryonic development. Some other studies previously reported that the NO/cGMP pathway was involved in the motility, invasion, and migration of trophoblast or smooth muscle cell (38, 39). An important finding is that L-NAME inhibits murine preimplantation embryo development in the blastocyst stage in which the embryo was going to distend and differentiate. This suggests that the inhibition of NO/cGMP pathway alter blastocysts' motility and migration, which might inhibit the normal embryonic growth.

Third, several studies have recently demonstrated that NO plays important roles in the reproductive system, such as regulating the release of LH-releasing hormone (LHRH), the zona pellucida–binding ability of human spermatozoa, and sperm mobility (9–12). Recent reports have also demonstrated that eNOS and iNOS expression was differentially regulated during the reproductive cycle (40, 41). The effects of NO on local vasodilation, control of trophoblastic invasion, and the prevention of fetal rejection in early pregnancy have been shown (12). Steroid hormones, inflammatory response, cytokines, and pregnancy influence both NOS expression and NO generation in female reproductive tract. Alterations in NO production could affect the development of embryos, especially during early stages of pregnancy (16, 19). According this evidence, we suggest that embryo-secreted NO plays an essential role in assisting the local vasodilation and hormone or cytokines regulation in the endometrium.

We demonstrated that normal embryonic development required NO production by the embryo itself, although several previous reports indicated that mice that are deficient in one isoform of NOS are viable (42–44). As mentioned in Gouge (16), it is possible that several different isoforms of NOS are present in murine embryos and can therefore compensate for the lack of only one isoform during development. We also showed that at least two isoforms of NOS (iNOS and eNOS) were both expressed in normal mouse embryos. In our study, the eNOS and iNOS immunostaining in the two-cell, four-cell, morula, and blastocyst stages were examined, but only blastocyst showed more significantly immunostaining.

NO has been proved to cause cytostatic and cytotoxic effects in many systems (18, 19, 45). Four days after the incubation with 10-μM SNP, 87.5% of embryo degeneration was observed. Many of the abnormal embryos after SNP treatment could be due to apoptosis, the process of programmed cell death. The possible linkage between abnormalities of the blastomeres and apoptosis was investigated by using two detection methods for cells undergoing apoptosis. Detection of phosphatidylserine exposure was performed using annexin V; the chromosomal breakdown after the nuclear collapse of apoptotic nuclei was tested using the TUNEL assay. TUNEL assay and annexin-V/propidium iodide specific-staining techniques provided the evidence that the NO donor, SNP (10 μM), induced embryo apoptosis with typical stages of chromatin condensation, cell shrinkage, and fragmentation.

Nitric oxide reacts rapidly with superoxide to form highly toxic peroxynitrite ($ONOO^-$). Both NO and $ONOO^-$ have a demonstrated ability to directly damage DNA (25, 29). These could trigger the p53-dependent or -independent apoptotic cell death pathways and subsequently induce cyto-

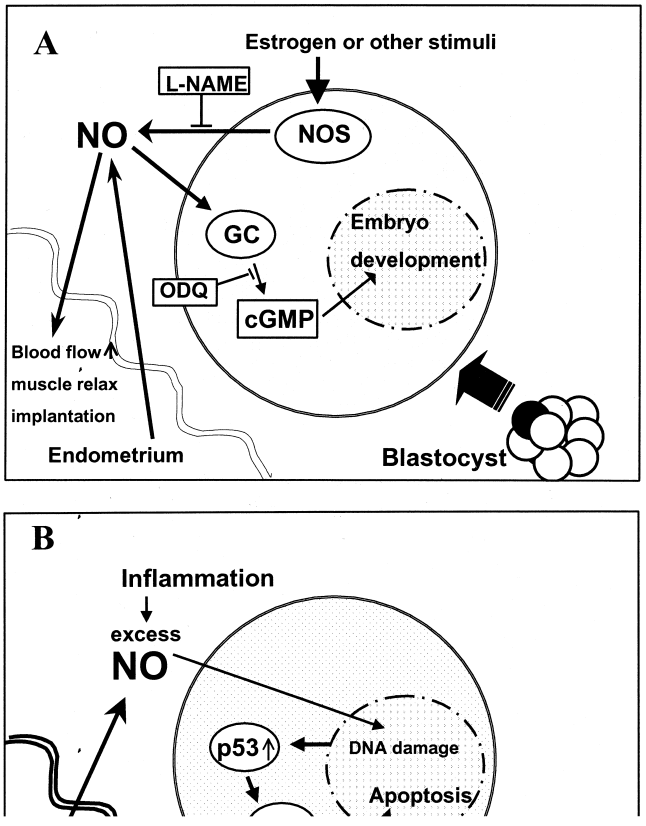
chrome *c* release from mitochondria, activate caspases or related proteases and cleavage of poly (ADP-ribose) polymerase, induce chromatin condensation, and result in DNA fragmentation (46, 47). The mechanism of this NO-induced embryo apoptosis remains to be determined. Although NO serves as a key signaling molecule in physiological processes, excessive or unregulated NO production has been shown to be a contributing factor to pathophysiological conditions, including vascular shock, stroke, diabetes, neurodegeneration, arthritis, chronic inflammation, and other lethal or debilitating diseases in human beings (3). Ota and colleagues found that both iNOS and eNOS expression were increased in endometriosis and adenomyosis and suggested its correlation with chronic inflammation and early embryo loss (17).

Previous reports indicated that products of human mononuclear phagocytes might contribute to the infertility associated with endometriosis (48). It appears that chronic stimulation of macrophages in the peritoneal cavity provokes constitutive release of large quantities of free-reactive oxygen products in women with endometriosis. This may occur secondary to the accumulation of activated monocytes into the peritoneal cavity (48). Macrophages are able to release NO as a defense mechanism (49). In contrast, up-regulation of iNOS, capable of producing excessive NO level, is implicated in tissue injury (50). The other important finding of this study was the first reported evidence for NO-induced apoptosis in the fragmented embryos. The therapeutic potential of selective inhibitors of NO formation is thus anticipated.

According to other reports and our own, these data suggest that NO might be involved in the modulation of embryo development (16, 19). However, the molecular mechanism for the NO-regulated embryo development remains unclear. Our findings demonstrate that cGMP reversed L-NAME-induced arrestment of embryo growth. ODQ is a selective soluble guanylyl cyclase inhibitor that was used to further determine the role of cGMP in mediating the embryo development regulated by NO. The data from the present study show that ODQ concentration dependently inhibited the embryo development at the concentrations of 1 to 100 μM. Cyclic GMP had shown no effect in embryo apoptosis when the concentration of SNP was in the range of 0.1–10.0 μM. The present study demonstrates that the cGMP pathway was involved in the signal transduction of NO-regulated embryo development but that embryo apoptosis induced by high concentration of NO was cGMP independent. Further studies are required to determine whether NO-induced embryo apoptosis is through the p53/Bax pathway. In conclusion, we provide the first evidence that the cGMP pathway was involved in the NO-regulated embryo development and that high-concentration NO induced embryo apoptosis in a cGMP-independent pathway. These results demonstrated that up-regulation of the expression of p53 and Bax might be involved in the NO-induced apoptosis. On the basis of the findings of the present and previous studies (16, 19, 21, 22), we assumed that the mechanisms of NO played a role in the regulation of early embryo development (Fig. 6A). The infertility associated with infection and endometriosis could be related to diminution of embryo development, induction of embryo apoptosis, and embryo loss through the production of excess NO in the reproductive tract (Fig. 6B).

FIGURE 6

Hypothetical model for the role of NO in embryo implantation, in which NO/cGMP pathway may increase blood supply and muscle relaxation in the endometrium and also be involved in embryo development (**A**). In the patients with endometriosis, the inflammatory cells may produce excess NO to induce embryo apoptosis through the p53/Bax pathway (**B**).



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