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The Effect of Nitric Oxide on Mouse Pre-Implantation Embryo Development and Apoptosis

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ABSTRACT

Clinical studies have shown that in pathological conditions such as endometriosis and reproductive tract infection (male and female) there is an activation status of macrophages that produce large quantities of nitric oxide (NO) in addition to other effector molecules. Large amounts of NO may have embryotoxic roles and produce infertility. This study was designed to evaluate the effect of different concentrations of NO on mouse pre-implantation embryo development *in vitro*. Mouse embryos (2-cell stage) were cultured in media containing different concentrations of sodium nitroprusside (SNP), an NO donor, or L-arginine methyl ester (L-NAME), an NO syntase (NOS) inhibitor. At the end of culture, cell apoptosis was studied by the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) technique. The results showed that development of preimplantation embryos were inhibited by high concentration of SNP (1 and 10 µM). In contrast, 0.1 µM of SNP stimulated the embryo development. Similarly, the inhibition of NO by NOS inhibitor resulted in the dose-dependent inhibition of embryo development, but the addition of 0.1 µM SNP with L-NAME reversed the inhibitory effect of L-NAME. TUNEL technique showed that high concentration of NO could induce apoptosis in the embryo, but at low concentration, it decreased apoptotic cell death. *Iran. Biomed. J. 7 (3): 107-111, 2003*

Keywords: Apoptosis, Nitric oxide (NO), Pre-implantation embryo

INTRODUCTION

Nitric oxide (NO) is an important mediator of many biological functions, and has beneficial effects when generated within physiological limits. But, when it was generated in large quantities for longer time, as during infection and immunological reactions, can cause cytotoxic effects [1]. NO is produced when the various isoforms of enzyme NO synthase (NOS) catalyze the oxidation of L-arginine to L-citrolline [1]. Inducible NOS (iNOS) is induced in a variety of cells, including macrophages, and other cell types by exposure to cytokines and inflammatory factors [2]. These reactions produced large amounts of NO, which contribute to the host defense, as well as killing the cells in the regions [3-5]. Large amounts of NO can promote breakage of DNA strands, which can initiate apoptosis and cause cell death in various cell types [6-8].

It has been suggested that NO has important physiological roles in a variety of reproductive processes such as follicular development, ovulation and spermatogenesis [9]. Some investigations have indicated that various isoforms of NOS are expressed in the oocyte, early preimplantation embryo, and uterus during pregnancy, and NO production at this time has an important role on embryo development and implantation [8-12]. But, other studies have shown that excess NO production or exogenous NO may have a role in embryo toxicicity [13, 14]. Clinical studies have shown that pathological conditions including endometriosis and reproductive tract infection (male and female) are associated with infertility [8, 15]. In both of these pathological conditions there is an increase in the

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number and activation status of macrophages in peritoneal fluid and in the reproductive tract [15, 16]. These activated cells could produce large quantities of NO in addition to other effector molecules [8]. High concentrations of NO could potentially inhibit embryo development and implantation and increase apoptosis of the embryos.

This study was designed to investigate the role of NO in controlling the development and the apoptosis of preimplantation mouse embryos.

MATERIALS AND METHODS

Embryo collection and culture. NMRI female mice at 5-6 week of age were super ovulated by 10 IU of human menopausal gonadoterophin (HMG) followed 48 h later by 10 IU of human chronic gonadotrophin (hCG) intraperitoneally. The female mice mated with adult male of the same strain immediately after the injection of hCG and then checked for mating the following morning. The mated female mice were killed by cervical dislocation 48 h after the hCG injection and the oviducts were removed. The 2-cell embryos were flushed from the oviducts and placed randomly in groups of 25-30 embryos in a drop of the different test media under light mineral oil at 37ºC in 5% CO_2 in air and then cultured for 96 hours. The experiment was repeated 5 times and finally 125-150 embryos were examined in each group.

Culture Media. The culture medium in control group was α-MEM containing 4 mg/ml bovine serum albumin (BSA fraction V; Sigma). In the treatment groups, different amounts (0.1,1 and 10 μM) of sodium nitroprusside (SNP, a NO donor) or L-arginine methyl ester (L-NAME, NOS inhibitor) were added to the α-MEM. To investigate the requirenment of NO for embryo development, in one group, the media containing 10 μM L-NAME was changed to control α-MEM after 24 h culture. To find the direct evidence whether the NO is essential for embryo development, in another group, 0.1 μM SNP was added to the medium containing 10 μM L-NAME. The medium for all groups was freshly prepared and replaced every 24 h throughout the study.

Embryo development. After 96 h culture, embryo development was assessed using an inverted microscope and the number of blastocysts and fragmented embryos were recorded.

Apoptosis Detection. To analyze the status of chromatin in embryos, we used a combined technique for simultaneous nuclear staining and TUNEL was used by modified method of Brison and Schultz [17]. Briefly, embryos were fixed at 4ºC overnight in 3.7% para formaldehyde diluted in PBS. Following fixation, they were washed 4 times in PBS/PVP, permeabilized in PBS containing 0.1% Triton-X100 for 1 hour and incubated in flurescein conjugated- dutp and TdT (TUNEL reagents), (Boehringer Mannheim, Tokyo, Japan) in an incubator at 37ºC and 5% CO_2 in air for 1 hour again. Positive controls were incubated in deoxyribonucleare I (Dnase I, 50 mg/ml; Sigma) at 37ºC for 20 min and 5% CO^2 in air and negative controls were incubated in fluorescein-dutp in the absence of TdT. After TUNEL, the embryos were washed 3 times in PBS/PVP and incubated in RNAse (50 mg/ml, Sigma) at room temperature for 60 min. again, they were incubated in PBS containing propidium iodide (PI, 50 μg/ml) at room temperature for 1 hour to label all nuclei.

After the incubation, they were washed 4 times in PBS/PVP and mounted on a glass slide and examined in whole mounts under a fluorescence microscope with an excitation filter of 460-490 nm and a barrier filter of 514 nm. The number of total cells and nuclei labeled by TUNEL were counted and for each blastocyst the incidence of TUNEL positive nuclei was calculated as percentage of the total cell number.

Statistical analysis. Differences among the means of development percentage in different culture media and differences in apoptotic cell indices were compared by one-way analysis of variance (ANOVA) coupled with Dunett 3 Post Hoch test using SPSS version 10.

RESULTS

The *in vitro* development of 2-cell mouse embryos after 96 hours culture in control and treated groups is summarized in Table 1. The SNP at concentrations of 1 μM and 10 μM significantly inhibited embryo development to the blastocyst stage in a dose-dependent manner (p<0.001). But the difference between the SNP (0.1 μM) group and control was not significant. Similarly, L-NAME significantly inhibited the embryo development to the blastocyst stage in a dose-dependent manner (p<0.001).

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Table 1. Two-cell stage embryo development after 96 hours culture in control (α-MEM*)* and treatment media.

Groups	Total number of two-cells embryo	Total number of blastocyst (% blastocyst ± SD)	Fragmented embryos (% fragmented ± SD)
Control (α-MEM)	137	115 (84.00 ± 2.60)	15 (13.80 ± 3.64)
SNP (0.1 μM)	138	122 (88.49 ± 2.50)	7 (5.00 ± 1.76)
SNP (1 μM)	139	62 (44.63 ± 2.00)	70 (50.00 ± 2.40)
SNP (10 μM)	131	0 (0)	116 (88.00 ± 3.00)
L-NAME (0.1 μM)	132	69 (52.25 ± 3.47)	59 (44.77± 6.44)
L-NAME (1 μM)	131	46 (35.18 ± 3.55)	61 (46.66 ± 4.00)
L-NAME (10 μM)	143	13 (9.00 ± 3.70)	74 (51.00 ± 3.80)
L-NAME (10 μM) (replaced with α-MEM after 48 h)	138	93 (67.50 ± 4.00)	25 (18.00 ± 4.00)
L-NAME (10 μM) + SNP (0.1 μM)	138	78 (56.50 ± 2.00)	52 (38.00 ± 3.00)

In the group that medium contain L-NAME and replaced with pure α-MEM after 24 hours, there were no significant differences in the the development of blastocyst stage when compared with the control group, but there were significant differences when compared with the embryos cultured in medium containing 10 μm L-NAME for 96 hours (p<0.001). In the group that 0.1 μM SNP was directly added to the medium containing 10 μML-NAME, the result indicated that the inhibitory effect of 10 μM L-NAME on embryo development could be rescused by 0.1μM SNP (%56.5 ± 4 blastocyst after 96 h culture).

The percentage of degenerated embryos in the SNP and L-NAME groups was significantly increased in a dose-dependent manner compared with the control and SNP (0.1 μM) groups (P<0.0001). However, the percentage of degenerated embryos was significantly decreased in the group L-NAME (10 μM) which after 24 h culture replaced with α-MEM or the group which added 0.1 μM SNP to the medium containing 10 μM L-NAME (P<0.001).

The proportion of the apoptotic cells in embryos cultured in one and 10 μM SNP was significantly higher than those cultured in control or 0.1 μM SNP (P<0.001) (Fig. 1). However, there were no significant differences among the other groups (L-NAME groups or the group which added 0.1 μM SNP to the medium containing 10 μM L-NAME) with control (Fig. 2).

DISCUSSION

Studies of the effects of NO on embryo development have produced several contradictory results. Some studies indicated that NO plays an

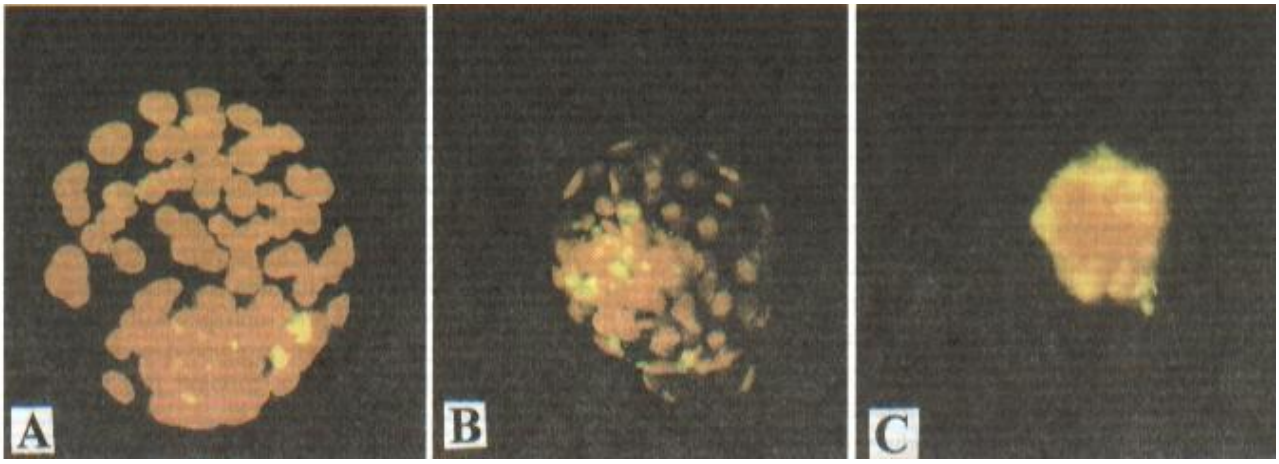

Fig. 1. (A), A blastocyst from control group after TUNEL. The fragmented nuclei that labelled by TUNEL are shown as bright yellow and the normal nuclei are shown in red. (**B),** A blastocyst from SNP (1μM). The proportion of apoptotic (bright yellow) nuclei are significantly higher than control; **(C),** A fragmented embryo from SNP (10 mM) group. Fragmented nuclei are labelled by TUNEL indicated that excessive nitric oxide kills cell through apoptotic cell death.

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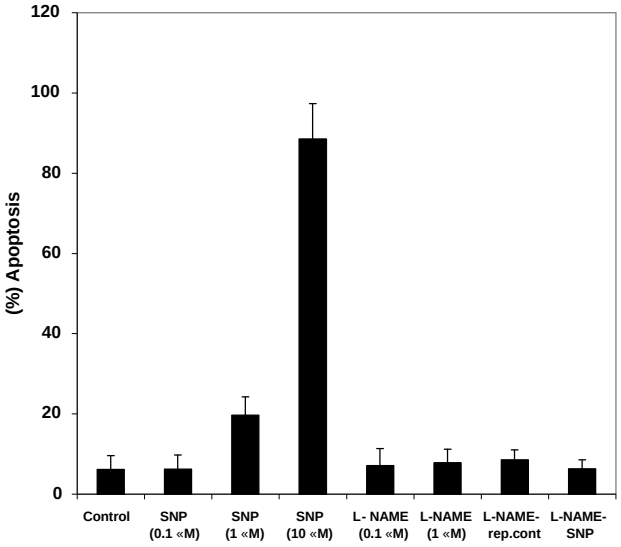

Fig. 2. Effects of SNP and L-NAME on apoptosis index in preimplantation embryos after 96 h culture. Among all groups, the SNP groups (1 and 10μM) showed significant differences with control and other groups ($p< 0.0001$).

important role in the regulation of fertility [1, 8, 9, 11]. Also, production of NO by preimplantation embryo and female reproductive tract is essential for embryo development and implantation. Some other studies indicated that NO, especially exogenous, inhibited embryo development and caused infertility [6, 7, 8, 13]. For example, Dong *et al*. [18] demonstrated the presence of various isoforms of NOS in several female reproductive organs, where its expression is differentially regulated during the reproductive cycle, suggesting that NO may be involved in the modulation of female reproductive function. In addition, other studies have supported an involvement of NO in normal embryo development [9-11]. However, some contro-versial reports indicated that high levels of NO, especially exogenous, causes delay in embryo development and increases apoptosis in embryonic cells [9, 13, 14, 19]. These results are completely compatible and the variance attributable to the means of NO introduction, the concentration of NO in the sample, and to the time frame over which the experiments were conducted.

Our results support Kazuo [9] and Couge [11] findings and demonstrate that the normal embryo development required NO production by embryo itself. The block of NO production by an inhibitor diminishes embryo development and induces apoptosis in embryonic cells. However, some of the previous reports indicate that exogenous NO suppresses mouse preimplantation embryo development at all concentration [9, 13]. Our results demonstrate that low concentration of NO (0.1 μM of SNP) enhances preimplantation mouse embryo development, but high concentration of SNP causes delay in embryo development and increases apoptosis in the embryonic cells. On the other hand, NOS inhibitor inhibits embryo development in a dose-dependent manner. This inhibitation of development is overcame by the addition of SNP to the medium containing L-NAME.

There was a significant difference between embryonic developmental rate and apoptosis in embryos cultured in the medium containing both an inhibitor and a NO donor. These findings indicate that a moderate amount of NO is essential for embryo but its excessive amounts is harmful. The over production of NO by embryo or other cells in response to the stimuli has deleterious effects on the pre-implantation embryos and damages embryonic cells through apoptotic cell death. Developmental damages excessively induced by excess NO may be the reason for the inflammatory disease such as endometriosis that induces early embryo loss and leads to the lower pregnancy rates [6]. Conversely, some other diseases such as diabetes, which decrease the NO production in the reproductive tract [20, 21] also diminishes embryo development and induces apoptosis in the embryonic cells. Therefore, the pathological conditions that change NO production in female reproductive tracts can cause infertility.

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