Full Paper

Increases in Serum Nitrite and Nitrate of a Few-Fold Adversely Affect the Outcome of Pregnancy in Rats

Tsuneo Inoue^{1,2}, Muneshige Kaibara^{2,*}, Yasuko Sakurai-Yamashita², Masahiro Kawano³, Tadayuki Ishimaru¹, and Kohtaro Taniyama²

Departments of ¹Obstetrics and Gynecology and ²Pharmacology, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan

³Tanaka Clinic, 17-12 Honmachi, Iizuka, Fukuoka 820-0042, Japan

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Abstract. The objective of this study was to evaluate serum nitrite and nitrate (nitrite/nitrate) concentrations that affect adversely pregnancy outcome. Pregnant rats, from day 2 to day 8 of pregnancy, were daily given subcutaneously several doses (5, 10, and 30 mg/rat) of diethylenetriamine-nitric oxide (DETA/NO). Serum nitrite/nitrate concentrations were measured using an HPLC system. Serum nitrite/nitrate concentrations increased dose-dependently with DETA/NO. Effects of DETA/NO on pregnancy outcome were assessed on day 14 of pregnancy. In rats given 5 mg DETA/NO, there was a significant increase in serum nitrite/nitrate concentrations (49.2 vs 24.6 $\mu\text{mol/l}$, $P < 0.001$), and both placental weight and fetal weight decreased compared to control rats. Macroscopic bleeding in placenta was frequently observed in rats given DETA/NO. We further studied effects of DETA/NO on cultured trophoblastic BeWo cells. DETA/NO added to the culture medium increased nitrite/nitrate concentrations in the medium in a dose-dependent manner. Nitrite/nitrate concentrations in the medium over four times the concentration of control decreased progesterone in the medium at 24 h after the application of DETA/NO. The hormonal secretion was not affected by DETA only. This study shows for the first time nitrite/nitrate concentrations affecting adversely pregnancy outcome and function of the trophoblastic cells.

Keywords: nitric oxide (NO), pregnancy, HPLC, diethylenetriamine-NO

Introduction

Nitric oxide (NO) is an important mediator in many physiological and pathological processes in various tissues (1). NO plays a significant role in the process of reproduction (2). It regulates the function of ovary and oviduct, contractility of uterine smooth muscle cells, uterine vascular tone, sperm motility, and fertilization. During pregnancy NO regulates umbilical-placental vascular resistance (3, 4) and inhibits contractility of uterine smooth muscles (5, 6). NO production in the uterus is accelerated during pregnancy (2). Inhibition of NO synthesis during pregnancy causes clinical signs similar to those of preeclampsia, such as an elevation of systolic blood pressure, proteinuria, a decrease in

weight, and an increase in mortality rate of fetuses (7).

In pathological conditions associated with infertility, such as endometriosis and reproductive tract infection (8, 9), there is possibility that NO concentrations increase in the reproductive tissues. In these conditions, macrophages in peritoneal fluid and in the reproductive tract are activated (10–12). The activated macrophages can release large quantities of several cytokines and NO, and thus in part, the increased NO may cause infertility in these conditions. Barroso et al. reported that exogenously applied NO inhibited development of embryos in vitro and implantation of embryos in vivo in mice, although serum levels of NO or NO-derived metabolites were not measured (13). Clinical studies have shown that, in pregnant women with a history of recurrent miscarriage, serum levels of nitrite/nitrate were slightly elevated compared to the case of normal

*Corresponding author. FAX: +81-95-849-7048
E-mail: mkaibara@alpha.med.nagasaki-u.ac.jp

pregnancy (14). However, it is not known whether the slightly increased nitrite/nitrate causes the miscarriage, since there are no reports concerning serum nitrite/nitrate levels which affect adversely pregnancy outcome.

In this study, we measured serum nitrite and nitrate (nitrite/nitrate) concentrations in pregnant rats given an NO donor and evaluated effects of NO on pregnancy outcome. We also examined effects of NO on human trophoblastic BeWo cells, which have hormonal functions of placental tissues, and measured nitrite/nitrate concentrations in culture medium. We now show nitrite/nitrate levels that affect adversely pregnancy outcome in vivo and inhibit the release of hormone from the cells in vitro.

Materials and Methods

Materials

Diethylenetriamine-nitric oxide (DETA/NO) was obtained from Funakoshi Co. (Tokyo). DETA and penicillin-streptomycin were from Sigma Chemical Co. (St. Louis, MO, USA). Ham's F12 was from Life Technologies, Inc. (Grand Island, NY, USA). Fetal bovine serum (FBS) was from Intergen Co. (Boston, MA, USA). Other reagents used were from Nacalai Tesque Co. (Tokyo).

In vivo study

The study was approved by the Institutional Animal Care and Utilization Committee of Nagasaki University and its guidelines were followed during the experiments.

Animals: Adult Wistar rats, weighing 200 to 260 g, were bred on a 12-h-light and 12-h-dark schedule. The presence of a vaginal plug and sperm in the vaginal smear on the morning following mating was defined as day 1 of pregnancy. Systolic blood pressure was measured using with a pneumatic tail-cuff device. The measurement was made before mating, on days 2, 8, and 14 of pregnancy.

NO donor treatment and nitrite/nitrate measurement: DETA/NO, a spontaneously releasing NO donor of which the half-life is relatively long, was dissolved in saline solution. DETA/NO at 5, 10, and 30 mg/rat or saline solution was subcutaneously injected into pregnant rats every day from day 2 to day 8 of pregnancy. Blood was collected from the retro orbital vein at 4 h after injection of saline or DETA/NO to measure concentrations of nitrite (NO_2^-) and nitrate (NO_3^-) in the serum before mating and on days 2, 8, and 14 of pregnancy. The serum samples were passed through a 0.22- μm membrane filter (MILLEX-GV; Millipore Co., Bedford, MA, USA) and stored in -80°C

until assay. For detection of nitrite and nitrate we used an automated NO detector-HPLC system (ENO-10; Eicom Co., Tokyo), as described elsewhere (15). Briefly, nitrite and nitrate in the sample were separated on a reverse-phase separation column packed with a polystyrene polymer, and nitrate was reduced to nitrite in a reaction column packed with copper-plated cadmium filings. Griess reagent was added on-line, and absorbance at 540 nm was measured using a flow-through spectrophotometer. Nitrite and reduced nitrate were quantified separately. The nitrite/nitrate concentration was the sum of nitrite and reduced nitrate.

Pregnancy outcome: On the day 14 of pregnancy, animals were dissected under anesthesia with pentobarbital and the uterus was checked for each implantation site, placenta, and fetus. We measured the weight of both placenta and fetus.

In vitro study

Cell culture, measurement of NO, progesterone, and human chorionic gonadotrophin (hCG) in media: BeWo cells, a trophoblastic cell line obtained from JCRB Cell Bank (Tokyo), were maintained in serial monolayer culture in Ham's F-12 supplemented with 10% FBS, 50 U/ml penicillin and 50 $\mu\text{g}/\text{ml}$ streptomycin at 37°C in humidified 5% CO_2 . The cells were cultured to near confluence into 6-well tissue culture plates (Asahi Technoglass Co., Tokyo) in 2 ml of the medium containing 1×10^5 cells/ml per well. After 24-h with DETA/NO or DETA, the media were collected and stored at -80°C until assay. Nitrite/nitrate concentrations in the media were measured using the method described above. Phenol red, which has a maximum absorbance similar to nitrite, in the media did not interfere with the measurement of nitrite/nitrate, since the reverse-phase column separated phenol red from nitrite and nitrate.

The cells were cultured into 24-well tissue culture plates in 1 ml of Ham's F-12 supplemented with 10% FBS, the penicillin-streptomycin containing 1×10^5 cells/ml per well. Twenty-four hours later, the media were replaced with Ham's F-12 supplemented with the penicillin-streptomycin, and we added DETA/NO or DETA. After additional 24-h incubation, the media were stored at -80°C until the hormone assay. Concentrations of progesterone and hCG in the media were measured in chemiluminescent enzyme immunoassays using an automated, quantitative immunoassay analyzer "Immulyze system" (DPC Inc., Los Angeles, CA, USA). The cells were washed with PBS, and solubilized in 0.15 mol/l NaOH and 0.5 mg SDS/ml at room temperature and the protein assay was done according to Lowry. Hormone concentrations were ng/ml per mg

for progesterone and mIU/ml per mg for hCG.

Cell proliferation assay: Proliferation of the cells was evaluated by WST-1 assay using a Cell Counting Kit (Dojindo Laboratory Co., Kumamoto). WST-1 assay was done according to the manufacturer's instructions. Briefly, BeWo cells were plated into 96-well tissue culture treated flat-bottom plates (Asahi Technoglass Co., Tokyo) in 100 μ l of medium containing 1×10^4 cells per well. The WST-1 solution was freshly prepared at a concentration of 5 mmol/l WST-1 and 0.2 mmol/l 1-methoxy-5-methyl-phenazinium methyl sulfate in 20 mmol/l HEPES buffer (pH 7.4). A 10- μ l aliquot of WST-1 reaction solution was added to each well, and plates were incubated for an additional 4 h. The plates were read in a microplate reader using a test wavelength of 450 nm and a reference wavelength of 690 nm.

Statistical analysis

The data are expressed as the mean $\pm$ S.E.M. For statistical analyses, we used either the unpaired *t*-test or one-way analysis of variance (ANOVA) followed by Fisher's multiple-comparison test. Statistical significance was set at $P < 0.05$. Statview software (SAS Institute Inc., Cary, NC, USA) was used for statistical analyses.

Results

In vivo study

We measured the serum nitrite/nitrate concentrations at selected times after subcutaneous injection of saline or DETA/NO (Fig. 1A). The concentrations at 4 h after injection of DETA/NO on days 2 and 8 of pregnancy were significantly higher in the rats given NO donor than in the rats given saline, and the increase was dose-dependent on DETA/NO. Averaged values of serum NO concentration calculated from days 2 and 8 were 24.6 ± 1.6 , 49.2 ± 6.9 , 72.6 ± 16.9 , and 191.6 ± 26.0 μ mol/l for rats given saline and 5, 10, and 30 mg/rat of DETA/NO, respectively. On day 14 of pregnancy, the day is 6 days after the end of the DETA/NO injection, the elevated serum nitrite/nitrate concentrations by DETA/NO injection reverted to the saline-injected level. In rats given saline, there were no significant changes in serum nitrite/nitrate concentrations during the course of pregnancy.

Systolic blood pressure of rats given DETA/NO or saline before mating, on days 2, 8, and 14 of pregnancy is presented in Fig. 1B. DETA/NO dose-dependently and significantly decreased systolic blood pressure in the DETA/NO administered rats compared to rats given saline. On day 14 of pregnancy, the decreased systolic blood pressure reverted to the saline-injected level.

Fig. 1. Serum nitrite/nitrate concentrations (A) and systolic blood pressure (B) on pre-mating, days 2, 8, and 14 of pregnancy in vehicle-treated (control, $n = 8$) or diethylenetriamine-nitric oxide (DETA/NO)-treated rats ($n = 6$ for 5 mg/rat, $n = 5$ for 10 mg/rat, $n = 5$ for 30 mg/rat). Each bar graph represents the mean $\pm$ S.E.M. * $P < 0.001$, compared to the control (multiple comparison).

The effects of different doses of DETA/NO on pregnancy outcome are summarized in the Table 1. There were no significant differences in the number of implantation sites between rats given saline and DETA/NO. In rats given DETA/NO, there were implantation sites having macroscopic bleeding in placenta, whereas there were no implantation sites having macroscopic bleeding in placenta in rats given saline. The mean weights of both the fetus and the placenta were significantly decreased in rats given DETA/NO compared to the rats given saline.

In vitro study

We measured nitrite/nitrate concentration in the medium at 24 h after the addition of DETA/NO or DETA into the medium. Nitrite/nitrate concentrations

Table 1. Effects of diethylenetriamine-nitric oxide (DETA/NO) on pregnant outcome

DETA/NO (mg/rat)	Number of rats	Number of implantation sites/rat	% of bleeding in placenta/rat	Weight of placenta (mg)	Weight of fetus (mg)
0	8	15 ± 2	0	80 ± 3	101 ± 3
5	6	9 ± 3	22.1 ± 8.4	69 ± 3*	87 ± 3*
10	5	14 ± 3	6.9 ± 2.8	67 ± 3*	86 ± 2*
30	5	12 ± 4	12.0 ± 7.5	72 ± 2**	91 ± 2**

Pregnancy outcome was assessed on day 14 of the pregnancy. % of bleeding in placenta: percentage of implantation sites having macroscopic bleeding in placenta. Results represent the mean ± S.E.M. * $P < 0.01$, ** $P < 0.05$, compared to the values observed in control rats (unpaired test).

Fig. 2. Nitrite/nitrate concentrations in culture media after 24-h incubation with diethylenetriamine-nitric oxide (DETA/NO) or DETA. Nitrite/nitrate concentrations in media treated with 10^{-4} , 10^{-3} , and 10^{-2} mol/l DETA/NO were significant increased in a dose-dependent manner. * $P < 0.0001$, compared to the control (CNT) (multiple comparison).

in the medium without DETA/NO (DETA) was $13.1 \pm 0.1 \mu\text{mol/l}$. As shown in Fig. 2, when DETA/NO was added to the medium, there was an increase in nitrite/nitrate concentrations in the treatment with DETA/NO of over 10^{-4} mol/l (56.4 ± 2.9 , 435.2 ± 9.6 , and $5513.6 \pm 220.7 \mu\text{mol/l}$ for 10^{-4} , 10^{-3} , and 10^{-2} mol/l of DETA/NO, respectively), which was dose-dependent on DETA/NO. In the medium containing DETA at the concentrations corresponding to DETA/NO, there were no significant changes in nitrite/nitrate concentrations.

As shown in Fig. 3A, the concentrations of progesterone in the medium decreased significantly at concentrations of 10^{-4} , 10^{-3} , and 10^{-2} mol/l DETA/NO. With the treatment with DETA/NO at 10^{-4} , 10^{-3} , and 10^{-2} mol/l, the progesterone concentrations were 25.2%, 13.1%, and 26.5% of DETA, respectively. Any dose of DETA did not change the concentration of

Fig. 3. Effects of diethylenetriamine-nitric oxide (DETA/NO) on the secretion of progesterone and human chorionic gonadotrophin (hCG) from trophoblastic BeWo cells. A: DETA/NO significantly suppressed progesterone secretion at concentrations of 10^{-4} , 10^{-3} , and 10^{-2} mol/l in the trophoblastic cells. B: A significant and specific decrease in hCG was seen in the cells treated with 10^{-3} mol/l DETA/NO. * $P < 0.001$, compared to the control (multiple comparison).

progesterone in the medium. As shown in Fig. 3B, a significant decrease in hCG concentration was observed in the medium containing 10^{-3} or 10^{-2} mol/l DETA/NO. At the concentration of 10^{-2} mol/l, DETA also decreased the concentration of hCG in the medium.

As shown in Fig. 4, much higher concentration of DETA/NO but not DETA inhibited cell proliferation.

Fig. 4. Effects of diethylenetriamine-nitric oxide (DETA/NO) on the proliferation of trophoblastic BeWo cells. The cell proliferation was evaluated by WST-1 assays. The cell proliferation was suppressed in the cases of 3×10^{-3} and 1×10^{-2} mol/l DETA/NO. * $P < 0.001$, compared to the control (multiple comparison).

Relative values of the proliferation to those of control cells treated with DETA were 13.7% and 12.1% for 3×10^{-3} and 10^{-2} mol/l DETA/NO, respectively.

Discussion

Firstly, we determined the serum nitrite/nitrate concentrations in the rats given DETA/NO. We showed that the subcutaneously injected DETA/NO functioned as an NO donor because DETA/NO dose-dependently increased serum nitrite/nitrate concentrations and decreased blood pressure and their parallel interaction, although, in part, the serum nitrite/nitrate was derived from endogenous NO and nitrite/nitrate in the diet. In the present in vitro study, not DETA but DETA/NO increased concentrations of nitrite/nitrate in the culture medium in a dose-dependent manner, and DETA/NO inhibited progesterone secretion and cell proliferation. It has been shown that not DETA but DETA/NO inhibits ovarian progesterone secretion from cultured ovarian cells in vitro and embryo implantation in mice in vivo (13, 16). Taken together, DETA/NO plays the role of an NO donor in vivo and in vitro. The serum nitrite/nitrate concentrations elevated in the first day of DETA/NO injection was not further elevated by injection every day for 8 days. Thus, DETA/NO did not accumulate, although its half-life is relatively long (17).

In the present study, DETA/NO induced placental bleeding and decreased both fetal weight and placental weight, suggesting that NO attenuates the function of the placenta and inhibits the growth of the placenta and/or the fetus. These effects of DETA/NO on pregnancy outcome were observed at the serum

nitrite/nitrate concentrations of twofold or more over the control levels, indicating that a slight increase in NO could be a cause of miscarriage. We attempted to perform an in vitro study using trophoblastic BeWo cells to determine if excessive NO directly affects the cells. Trophoblastic cells are hormonally functional cells that synthesize hCG and progesterone as the placenta does (18, 19). These hormones have an important role in maintaining the pregnancy. It has been reported that NO attenuates the release of hCG from trophoblastic cells (20) and steroid genesis in the reproductive system (2). We measured nitrite/nitrate concentrations in the culture medium. We found that increased nitrite/nitrate in the culture medium of over 4 times the level of the control inhibited the release of progesterone from the cells. Higher DETA/NO was required to inhibit the release of hCG than the release of progesterone in the medium, indicating that the steroid genesis is more sensitive to NO than hCG synthesis in trophoblastic cells. These in vivo and in vitro data suggest that few-fold increases in NO inhibit steroid genesis in placenta and restrain growth of placenta and fetus. In the present study, the effects of DETA/NO on pregnancy outcome were not dependent on the amounts of DETA/NO. It is well known that an application of an NO donor attenuates intrinsic NO synthase (21). It is possible that the NO synthesis in the reproductive tissues is suppressed by the excess NO applied exogenously. Therefore, the NO levels in the reproductive tissues may increase a little if the serum NO levels increase by the application of DETA/NO.

Although the nitrite/nitrate in the culture medium at concentrations comparable to in vivo study did not inhibit the proliferation of BeWo cells, much higher concentrations of nitrite/nitrate, approximately 100 times of the control level, inhibited the proliferation of BeWo cells, suggesting that high concentration of NO directly inhibits placental growth. In early pregnancy, cytotrophoblast cells invade the uterus and differentiate into uterine arterioles to anchor the fetus to the mother and create low resistance vessels that carry blood to the floating villi at the maternal-fetal interface (22). High concentration NO may suppress the invasion of trophoblast to the decidualized endometrium and myometrium, the result being the inhibition of implantation. Previously, Barroso et al. showed that DETA/NO inhibited implantation in mice in vivo (13), in which continuous application of DETA/NO ($5 - 20 \mu\text{mol}/\text{animal per day}$) using mini-osmotic pumps was given, and they found that the high concentration of DETA/NO resulted in a decrease in the number of implantation sites. Although we used $30 - 160 \mu\text{mol}/\text{animal per day}$ DETA/NO, there were no significant differences in

the number of implantation sites with any concentrations of DETA/NO compared to rats given saline, indicating that the inhibition of the proliferation of the trophoblastic cell is irrelevant to the adverse effects of DETA/NO on pregnancy outcome. The discrepancy might be due to the difference in the way of application of DETA/NO. We injected DETA/NO once a day subcutaneously. Continuous application of DETA/NO using the osmotic pump may result in an accumulation of DETA/NO because of its long half-life (17).

In summary, we showed for the first time the serum nitrite/nitrate concentrations that affected adversely pregnancy outcome in rats given an NO donor during the course of pregnancy. Our data suggest the clinical importance of monitoring serum nitrite/nitrate in women with a history of recurrent miscarriage.

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