Journal of Assisted Reproduction and Genetics, Vol. 20, No. 11, November 2003 (© 2003)

Assisted Reproductive Technologies

Embryonic Production of Nitric Oxide and Its Role in Implantation: A Pilot Study

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Submitted September 18, 2003; accepted October 1, 2003

***Purpose*:** To investigate the ability of human embryos to produce nitric oxide (NO) and correlate its production with embryo quality and pregnancy rate.

***Methods*:** Twenty-three women participated in the study and were submitted to controlled ovarian stimulation and intracytoplasmic sperm injection. Embryos were singularly cultured in medium microdrops of 50 μL and were replaced, by transcervical transfer, at the 2- to 6-cell stage. In the culture media of each embryo the NO production was assessed by monitoring the levels of its stable oxidation products (nitrites/nitrates).

***Results*:** All the 23 patients underwent embryo transfer. After microinjection 64 embryos were obtained. The mean number of transferred embryos was 2.61 ± 0.46 and the pregnancy rate was 26%. The mean nitrite/nitrate concentrations of culture medium of each embryo was significantly higher (5.88 ± 2.34 μmol/L) than in pure P-1 medium (0.81 ± 0.21 μmol/L; $p < 0.001$) demonstrating an embryonic secretion of NO. Comparing pregnant (7.34 ± 2.72 μmol/L) versus nonpregnant patients (5.53 ± 1.49 μmol/L; $p = 0.022$), the mean nitrite/nitrate concentrations were significantly higher. Furthermore, the best quality embryos of pregnant women produced significantly higher nitrite/nitrate concentrations than those of not pregnant patients.

***Conclusions*:** It seems that NO production in nidating embryos is increased and that it may be primarily associated with a better morphology and a better growth potential of developing embryos.

KEY WORDS: Embryo; ICSI; implantation; infertility; nitric oxide.

INTRODUCTION

Helping infertile couples to have healthy children is one of the primary tasks of assisted reproductive technologies. To fulfil this task, reproductive medicine constantly needs to obtain information on physiology and pathophysiology of reproduction.

In the last years, nitric oxide (NO) has assumed an important functional role in the physiology of several organs and systems (1). Nitric oxide is a labile and diffusible molecule that forms stable oxidized metabolites (nitrite/nitrate; NO_2^-/NO_3^-) detectable in many biological fluids. In vivo, NO is formed from L-arginine either by a constitutive calcium-dependent, or a proinflammatory cytokine-inducible, NO–synthase (NOS) (2). Although the precise role of NO has not been elucidated, it has been suggested that NO induces vasodilation, inhibits platelet aggregation and smooth muscle cells proliferation, acts as a neurotransmitter in the neural system and as a cytotoxic agent for the invading microbes (2,3). Apart from the above functions, NO may be directly involved in the physiology of reproductive organs. The NO participates in

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1058-0468/03/1100-0449/0 © 2003 Plenum Publishing Corporation

periovulatory modulation of ovarian blood flow (4–6) and seems to be involved in follicular maturation and ovulation (1,7). However, although the NO local synthesis within the uterus may be important for regulating endometrial, cervical, and myometrial activity (8), its role in embryonic implantation has not been fully explored.

Successful embryo implantation resembles an inflammatory reaction, with infiltration of leukocytes, vasodilation, and oedema (9) and it is strictly dependent on the synchrony between embryo development and the endometrial events that occur in the peri-implantation period of a fertile cycle (10). These local modifications are fundamental for the generation of the decidual tissue and involve the production of vasoactive agents such as prostaglandins, leukotrienes, platelet activating factor, and NO (11–14). A critical level of NO seems to be mandatory for the establishment of pregnancy. In fact, although Chwalisz *et al*. (15) demonstrated that NO is required for normal embryonic development, Barroso *et al*. (16) showed that high NO concentrations, in mice, may be cytotoxic and could alter either the embryo development in vitro and implantation in vivo.

The aim of this study was to investigate the ability of human embryos to produce NO and correlate the NO production with embryo quality and pregnancy rate.

MATERIALS AND METHODS

The study protocol was approved by the institutional Ethics Review Committee. All the 23 women attending the Bologna University Infertility Clinic who participated in the study provided their informed consent.

The mean (±SD) age of the patients was 35.2 ± 4.2 years (range 28–41), and the mean duration of infertility was 6.8 ± 3.2 years (range 3–11). All patients were selected from women who suffered from tubal infertility. All had regular menstrual cycles (28 ± 4 days), and their partners were fertile according to World Health Organization's standards (17). Patients with concurrent illness were excluded from the study. Other exclusion criteria included body mass index [BMI = weight (kg)/weight $(m)^2$] $\geq$30, endometriosis, ovarian functional cyst, polycystic ovarian syndrome, unilateral ovarian resection, or ovariectomy. Likewise patients who were heavy smokers (>10 cigarettes/day), and were hypertensive (systolic blood pressure >140 mmHg and/or diastolic pressure >90 mmHg) were excluded from the study. None of the women had received hormonal treatments for at least 4 months before the controlled ovarian hyperstimulation.

Controlled ovarian stimulation was achieved by an i.m. injection on day 20 of the cycle of GnRH agonist triptorelin (Decapeptyl 3.75; Ipsen, Milan, Italy) and, after pituitary desensitization (plasma estradiol concentration <100 pmol/L; ovaries with no follicles >5 mm in diameter and endometrial thickness <5 mm), i.m. administration of pFSH (Metrodin HP; Serono, Rome, Italy; 225 IU in the first 3 days of the cycle, then in an individually assessed dosage).

When at least two follicles $\geq$17 mm in diameter were present, pFSH was withdrawn and 10,000 IU hCG (Profasi; Serono) were administered i.m. On the same day: (a) peripheral blood was obtained and plasma estradiol concentrations were determined by radioimmunoassay (RIA; Radim, Pomezia, Italy); and (b) ultrasonographic assessment of endometrial thickness was performed using a 6.0-MHz transvaginal transducer (Versa Plus; Siemens, Milan, Italy).

Ultrasonographic oocyte recovery was performed transvaginally 35–36 h after hCG injection. The retrieved oocytes were classified as mature, immature, or atretic on the basis of the morphology and appearance of the oocyte cumulus–corona complex according to published criteria (18). To obtain standardized condition, up to four mature oocytes/patient were selected and submitted to intracytoplasmic sperm injection (ICSI). The oocytes selected for insemination were singularly placed, under oil (Mineral Oil; Medi Cult, Mollehaven, Denmark), in medium microdrops of 50 μL (IVF medium; Irvine Scientific, Santa Ana, CA) and incubated at 37°C in a 5% CO_2 atmosphere. After at least 3 h the eggs were briefly exposed to 40 IU/mL hyaluronidase (Medi Cult, Mollehaven, Denmark) and mechanically denuded (170–140–130 μm denuding pipette; Cook, Melbourne, Australia). The denuded oocytes were examined under an inverted microscope to accurately evaluate the presence of the first polar body (1st PB) and maintained in culture until microinjection.

After 2–3 days of sexual abstinence, semen samples were produced by masturbation, collected in sterile specimen cups and allowed to liquefy at room temperature. Semen volume, pH, sperm concentration, motility, and morphology were determined following the WHO guidelines for semen analysis (17). All semen samples were washed in Flushing medium (Medi Cult, Mollehaven, Denmark) by centrifugation at 200 × g for 10 min and treated utilizing a discontinuous gradient technique (Pure Sperm; Medi Cult, Mollehaven, Denmark).

Intracytoplasmic sperm injection was performed as described by Van Steirteghem *et al.* (19).

To avoid ethical, legal, and religious problems, the supernumerary oocytes were cryopreserved as previously described (20).

The fertilization check (presence of two pronuclei and of the 2nd PB) was performed 16–18 h after sperm injection. Zygotes and embryos were singularly cultured in medium microdrops of 50 μL (P-1 medium; Irvine Scientific, Santa Ana, CA) under oil.

To study the impact of embryo quality on NO production, the embryos were graded morphologically before replacement. The embryos were scored as follows: grade A, equal-sized blastomeres, no fragmentation; grade B, equal- or unequal-sized blastomeres, <20% fragmentation; grade C, equal- or unequal-sized blastomeres, 20–50% fragmentation; grade D, equal- or unequal-sized blastomeres, >50% fragmentation. Embryo transfer (ET) was performed 48 h after oocyte retrieval. Between one and four embryos were replaced at the 2- to 6-cell stage. Transcervical transfer was carried out using a Frydman catheter (SCS International; Genoa, Italy).

Vaginal progesterone (Esolut; Angelini, Rome, Italy) was prescribed as luteal phase support until the β-hCG assay was performed. A clinical pregnancy was diagnosed by ultrasonographic evidence of embryonic hearth activity.

Immediately after ET, the culture medium of each embryo was collected in Eppendorf cryotubes (SGA/Diasint, Florence, Italy) and stored at -20°C until NO^{-} was assayed.

Nitric oxide production was assessed by monitoring the culture media levels of stable oxidation products of NO metabolism (NO_2^-/NO_3^-). Since very little or no NO_2^- is normally found in the biological fluids, no attempt was made to differentiate between NO_2^- and NO_3^- amounts; hence, results were reported as NO_2^-/NO_3^-. Nitrites/nitrates were assayed using the Greiss reaction with previously described procedures (21,22). All samples were analyzed in duplicate. The NO_2^-/NO_3^- concentration in the P-1 medium (Irvine Scientific) of five different bottles were assayed in duplicate. The NO_2^-/NO_3^- intra- and interassay coefficient of variation were 5.6 and 6.8% respectively.

Statistical Analysis

A statistical analysis (SPSS software; SPSS Inc., Illinois, USA) was performed using the one-way analysis of variance with Bonferroni correction. The relationship between the parameters analyzed was assessed using the stepwise linear regression method. A probability of <0.05 was considered statistically significant. Data are presented as mean $\pm$ SD, unless otherwise indicated.

RESULTS

All the 23 patients underwent ET and completed the study. No cycle was cancelled either because a "poor response" (estradiol plasma levels <1100 pmol/L and/or <3 follicles recruited by cycle day 8) or because a risk of hyperstimulation syndrome (>15 follicles/ovary and/or estradiol plasma levels >9000 pmol/L).

During the controlled ovarian hyperstimulation, the mean number of pFSH ampoules we used was 45.4 $\pm$ 10.3 and the mean duration of pFSH treatment was 12.5 $\pm$ 2.1 days. On the day of hCG administration, the number of recruited follicles was 11.5 $\pm$ 3.3; the mean endometrial thickness was 10.4 $\pm$ 1.4 mm; and the mean estradiol plasma level concentrations was 7245 $\pm$ 1105 pmol/L. During ovum pick-up were collected 99 oocytes and among them 67 mature oocytes were submitted to ICSI. The remaining 33 oocytes were cryopreserved. After microinjection 64 embryos (fertilization rate = 95%) were obtained and their morphology allowed us to distinguish 16 (25%) grade A, 32 (50%) grade B, and 16 (25%) grade C + D embryos. The mean number of transferred embryos was 2.61 $\pm$ 0.46 and the pregnancy rate was 26% (6/23). The pregnancy rate per cycle, per patient, and per ET were coincident since all the patients underwent to ET. Five single and one twin (implantation rate = 10.9%) pregnancies were obtained and all resulted in normal live births.

The mean NO_2^-/NO_3^- concentrations of culture medium of each embryo was significantly higher (5.88 $\pm$ 2.34 μmol/L; range 2.59–14.32 μmol/L; 10th percentile = 4.02 μmol/L, 90th percentile = 8.49 μmol/L) than in pure P-1 medium (0.81 $\pm$ 0.21 μmol/L; $p \leq 0.001$) demonstrating an embryonic secretion of NO_2^-/NO_3^-. No significant differences were observed between the NO_2^-/NO_3^- secretion of grade A (5.66 $\pm$ 1.76 μmol/L), grade B (6.14 $\pm$ 2.15 μmol/L), and grade C + D (5.86 $\pm$ 2.71 μmol/L) embryos. However, comparing pregnant versus nonpregnant patients, the mean NO_2^-/NO_3^- concentrations of culture medium of each embryo was significantly higher (7.34 $\pm$ 2.72 μmol/L) in the first than in the second group (5.53 $\pm$ 1.49 μmol/L; $p = 0.022$). In addition, the best quality (grade A + B) embryos of pregnant women produced significant higher NO_2^-/NO_3^- concentrations

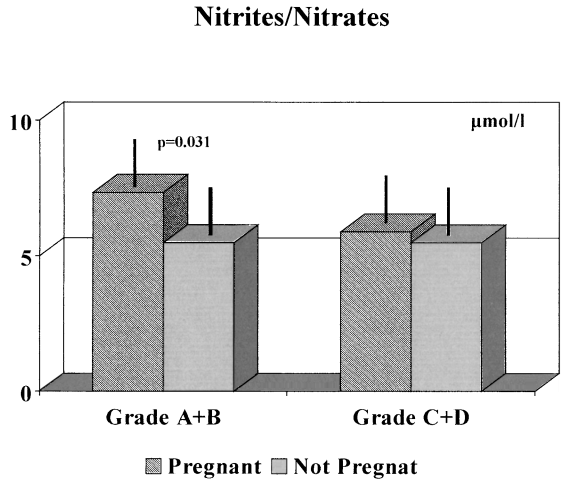

Fig. 1. The best quality (grade A + B) embryos of pregnant women produced significant higher NO_2^-/NO_3^- concentrations than those of not pregnant patients. No differences among the two groups were observed between grade C+D embryos.

than those of not pregnant (Fig. 1) patients. No differences among the two groups were observed between grade C + D embryos (Fig. 1). Furthermore, in seven cases, among the embryos belonging to the pregnant group, the NO_2^-/NO_3^- concentrations were above the 90th percentile and the transferred embryos were at the 6-cell stage. The endometrial thickness was not significantly different in pregnant and nonpregnant women.

No significant correlations were found between NO_2^-/NO_3^- concentrations, embryo grading, pregnancy rate, and estradiol circulating levels.

DISCUSSION

The embryonic nidation is a critical process when pregnancy occurs. Successful implantation of blastocyst is, in fact, due to the synchrony between the embryo development and the endometrial transformation into the decidua (an intense vascularized tissue that encapsulates the developing embryo). The factors responsible of the endometrial/decidual cell transformation are still poorly understood and seem to involve vasoactive agents such as prostaglandins, leukotrienes, platelet activating factor, and NO (11–14).

The role of NO in the nidation process has been fundamentally studied in the rodent model and an increased expression of NO synthase has been found at the implantation site on the first days of pregnancy (23). Furthermore, the administration of a Nω-nitro-L-arginine methyl ester, a NOS inhibitor, reduces the number of successfully implanted embryos and indicates that the generation of NO is a primary step for embryonic implantation (24–26). In addition, Gouge *et al.* (27) recently showed that NO is required for the normal embryonic mitotic division and that the inhibition of NO, by altering the cyclic nucleotide production, has detrimental effects on embryonic development. On the other hand, Barroso *et al.* (16) demonstrated that, in mice, elevated NO concentrations, inhibit both embryo development in vitro and implantation in vivo. The NO-induced cytostasis and cytotoxicity may be due to a specific inhibition of mitochondrial respiration and DNA synthesis.

From the above data in rodents it is evident that the successful establishment of pregnancy requires a critical level of NO at the nidation site and that increased NO levels may be deleterious to implantation. In humans there are scant data on the specific role of NO in embryo development, decidual transformation, and embryo implantation. However, indirect evidences confirm that, depending on its concentrations, NO can both inhibit or stimulate embryonic development and implantation (28,29).

In this study we demonstrated, for the first time in humans at the best of our knowledge, an embryonic secretion of nitrites/nitrates. Although the NO production did not result correlated with embryo grading, the mean nitrite/nitrate concentrations of culture medium was significantly higher in the best quality embryos of pregnant than nonpregnant women. We speculated that the embryonic morphology is an expression of its growth potential and that the loss of any correlation between embryo grading and NO production may only be due to the low number of studied embryos.

It is well-known that each embryo has its own developmental potential, and that few cleaved embryos are competent to implant after IVF and develop throughout gestation. Our data showed that, in seven cases belonging to the pregnant group, the NO concentrations were above the 90th percentile and the embryos were at the 6-cell stage. The results we derived from this pilot study, led us to speculate that NO production in nidating embryos is increased and that it may be primarily associated with a better morphology and a better growth potential of developing embryos.

The above results were further associated with no significant differences in circulating estradiol levels and endometrial thickness demonstrating the limited value of these parameters in the blastocyst implant.

Although it is reported that elevated NO concentrations have been found in pathologic cases in which implantation failures occur (endometriosis, recurrent miscarriages, etc.) (30,31), we think that, if comprised in a physiological range, increased NO may act at different sites to promote implantation. It could contribute to embryonic implantation by increasing: endometrial vascular permeability; prostaglandin mediated decidualization; programmed cell death; extracellular matrix degradation; and uterine relaxation (2,3,32,33).

Although further larger prospective studies are necessary to elucidate the complex factors that control the reciprocal signalling relationship between maternal and embryonic tissues, it may be concluded that NO is actively produced by the developing embryos and may be deeply involved in embryo nidation.

ACKNOWLEDGMENTS

The authors thank Dr Eleonora Porcu, Prof Stefano Venturoli, and the staff of Bologna University Infertility Clinic for their invaluable help and cooperation. They also thank Daniele Radi for expert assistance in laboratory assays.

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