Nitric oxide in blastocyst implantation in the rhesus monkey

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Abstract

Successful blastocyst implantation depends on the interaction between cells of maternal endometrium and conceptus, as well as adequate blood supply to the site of blastocyst implantation. Nitric oxide (NO) generally plays a significant role in the local regulation of vascular physiology in a variety of mammalian tissue systems, however, its role in blastocyst implantation and placentation in the primate is not known. The aim of the present study was to examine: (i) NADH-diaphorase activity and expression of three isoforms of nitric oxide synthase (NOS), namely endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS) in pre-implantation stage monkey embryos, morula ($n = 4$) and blastocyst ($n = 10$), as well as, in different compartments of conceptus and maternal endometrium at primary implantation sites during lacunar ($n = 6$) and villous ($n = 9$) stages of placentation in the rhesus monkey, and (ii) the potential anti-nidatory effect of vaginal administration of NOS inhibitor during the peri-implantation period of conception cycles in rhesus monkeys. Pre-implantation stage blastocysts exhibited marked NADPH-diaphorase activity along with immunopositive iNOS mainly in the inner cell mass. During the lacunar stage, marked eNOS expression was observed in cytotrophoblast cells lining the embryonic cavity. However, cytotrophoblast cells lining villi, forming columns, and constituting anchoring villi expressed all the three isoforms of NOS in villous placenta stage tissue. During the lacunar stage, eNOS and iNOS protein expressions were observed in epithelial and decidual cells of endometrium. As gestation advanced, mRNAs for all three isoforms of NOS were observed to increase in epithelial and decidual cells, however, with no marked change in protein expression. Vaginal administration of a NOS inhibitor (N^G -nitro-L-arginine methyl ester, L-NAME, 4, 6, and 8 mg/kg body weight or aminoguanidine, AG, 4 mg/kg body weight) during days 6 to 12 after ovulation resulted in pregnancy failure in a higher number of animals (L-NAME: 8 confirmed pregnancies in 25 animals; AG: 2 confirmed pregnancies in 8 animals) compared with control animals (5 pregnancies in 7 animals). It appears that NO may play an important role in the establishment of pregnancy in the rhesus monkey.

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Introduction

Nitric oxide (NO) is a multifunctional biomolecule that is produced by the enzyme nitric oxide synthase (NOS) (Moncada *et al.* 1991, Radomski 1995). There are three distinct isoforms of NOS; endothelial and neuronal types (eNOS and nNOS respectively) are constitutive and calcium-calmodulin dependent, while the third one is inducible NOS (iNOS), which is not dependent on calcium-calmodulin for its enzymatic activity (Berdeaux 1993). NO is involved in a number of physiological processes which include vasodilation, angiogenesis, platelet aggregation, leucocyte egression, macrophage activity, immune functions, connective tissue remodeling and smooth muscle activity (Moncada *et al.* 1991, Radomski 1995, Sooranna *et al.* 1995). Some of these events occur during the establishment of pregnancy, and the involvement of NO in mammalian embryogenesis and gestation

has been demonstrated in small laboratory animals (Chwalisz *et al.* 1999, Ota *et al.* 1999, Purcell *et al.* 1999, Gaglioti *et al.* 2000, Chen *et al.* 2001, Novaro *et al.* 2002). There is also evidence suggesting that human endometrial cells express NOS (Telfer *et al.* 1995, Tseng *et al.* 1996, Khorram *et al.* 1999) and that uterine NO may be involved during pregnancy in the human (Natuzzi *et al.* 1993, Yallampalli *et al.* 1994, Dong *et al.* 1996, Sladek *et al.* 1997). NOS has been localized in syncytiotrophoblast cells of term placenta (Conrad *et al.* 1993, Myatt *et al.* 1993), and in extravillous trophoblast cells (Martin & Conrad 2000), as well as, cytotrophoblast cells of the cell column of anchoring villi (Ariel *et al.* 1998) of the human placenta. However, there is no knowledge about the localization of NOS expression in cells of conceptus and maternal endometrium at the time of blastocyst implantation and initiation of placentation in the human and in the non-human primate. More over, it is not known

whether inhibition of NOS activity may inhibit the process of establishment of pregnancy in the primate. In the present study, we have examined: (i) the NOS expression in pre-implantation stage rhesus monkey embryos, morula and blastocyst, and (ii) the expression patterns of mRNA and protein of NOS isoforms in different cell types of maternal endometrium and conceptus at the primary implantation site of the rhesus monkey. Furthermore, we have examined the hypothesis that NO is important for the establishment of pregnancy with the use of NOS inhibitor, e.g. N^G -nitro-L-arginine methyl ester (L-NAME), or aminoguanidine (AG) (Joly *et al.* 1994) administered intra-vaginally during peri-implantation stage of potential conception cycles in rhesus monkeys.

Materials and Methods

Animal

Sexually mature, healthy and proven fertile female (4.5–5.8 kg) and male (6.5–8.5 kg) rhesus monkeys (*Macaca mulatta*) were used in this study. Monkeys were individually housed in cages having access to natural light and fed with standard pellet diet supplemented with fresh fruits, soaked gram, peanut, and water *ad libitum*. The study was carried out at the Primate Research Facility (PRF) of the All India Institute of Medical Sciences (AIIMS), New Delhi, India. The experiment was performed with the approval of the Ethics Committee for the Use of Non-Human Primates in Biomedical Research of the AIIMS.

Blood sampling and estimation of hormone levels

Every morning blood samples were collected by venipuncture from cycle day 8 onwards until the day of tissue collection or embryo recovery and were used to determine the peripheral serum levels of oestrogen, progesterone, and monkey chorionic gonadotropin (mCG), as described previously (Ghosh *et al.* 1997). Serum samples were kept at -20°C until hormone assays were performed.

Pre-implantation stage embryo

The methodological details for pre-implantation stage embryo recovery are given elsewhere (Ghosh *et al.* 1994). Briefly, female animals ($n = 18$) showing at least two consecutive cycles of normal length (26–32 days) were used in this part of the study, and were cohabited with males between days 8 to 16 of the ovulatory cycles. Success in insemination was assessed by daily microscopic examination of vaginal smear during the mating period. On day 6 after ovulation, as estimated from serum oestradiol-17 β and progesterone profiles, monkeys were laparotomized under ketamine hydrochloride (12 mg/kg body weight, i.m.; Parke-Davis, Mumbai, India) anaesthesia, and after checking the ovulation point the entire reproductive tract was flushed with Hank's balanced salt solution (pH 7.2) to retrieve embryos as described previously (Ghosh &

Sengupta 1991, Ghosh *et al.* 1994). Fourteen animals yielded pre-implantation stage embryos: four morulae, seven zona-encased blastocysts and three zona-free blastocysts. No embryo could be recovered from four animals despite clear ovulation and good insemination. Six embryos (two morulae, three zonal blastocysts and one zona-free blastocyst) were fixed in 4% paraformaldehyde in phosphate buffer (pH 7.4), dehydrated in a series of graded ethanol and embedded in London Resin White (EM Sciences, Washington, PA, USA) according to the protocol given by the manufacturer (Ghosh *et al.* 2000a). One micrometre sections were collected on glass slides and used for immunohistochemistry as described below. The other eight pre-implantation stage embryos were processed for NADPH-diaphorase reaction *in vitro* as described below.

Tissue collection and analysis of implantation sites

The methodological details are given elsewhere (Ghosh *et al.* 1999). Briefly, menstrual cycles were monitored by daily examination of vaginal swabs. Female monkeys ($n = 20$) showing at least two consecutive cycles of normal length (26–32 days) were used in this part of the study, and were cohabited with males from days 8–16 of ovulatory cycles. Success in insemination was assessed by daily microscopic examination of vaginal smear during the mating period. On estimated day of gestation (days 12 to 17), monkeys were laparotomized under ketamine anaesthesia as described above, and subjected to *in situ* perfusion fixation with 4% neutral-buffered paraformaldehyde. Uteri were then removed by hysterectomy. Each uterine specimen was quickly washed in cold phosphate-buffered saline (PBS) to remove adherent blood and was excised to expose the primary implantation site which was then placed in cold 4% paraformaldehyde fixative for 24 h at 4°C and then further processed through graded dehydration, clearing and embedding in paraffin (Ghosh *et al.* 1999). Based on light microscopic examination of haematoxylin stained paraffin sections, the implantation stages for each sample were documented based on earlier observations (Ghosh *et al.* 1999, 2000b, 2004, Dhara *et al.* 2001, Sengupta *et al.* 2003) and 15 samples were selected for further study. Implantation sites and associated fetal and maternal compartments were analysed using the zonation description given by Enders & King (1991). Immunohistochemical localization for cytokeratin, vimentin, and von Willebrand factor (vWF) was performed in successive sections to distinguish trophoblast cells, mesenchymal cells, stromal decidual cells and endothelial cells, respectively at implantation sites (Ghosh *et al.* 1999, 2000b, 2004, Dhara *et al.* 2001, Sengupta *et al.* 2003).

NADPH-diaphorase histochemistry

Eight embryos were processed for NADPH-diaphorase histochemistry according to the procedure described by

Nishikimi *et al.* (2001). Five recovered embryos were washed in PBS (pH 7.2) containing 0.2% Triton-X100 and subjected to incubation for 1 h at 37°C in Tris-HCl (0.1 mmol/l, pH 7.2) buffer containing β -NADPH (1 mmol/l) and nitroblue tetrazolium (0.2 mmol/l). Three embryos were used as the control in which the incubation was performed as above but no β -NADPH was added in the incubation medium. The embryos were washed quickly in buffer containing 0.2% Triton-X100 and mounted onto slides for visualization and documentation. All the chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA).

Immunohistochemistry

Immunohistochemistry staining was performed with buffered, paraformaldehyde-fixed, deparaffinised tissue sections (5 μ m) and London Resin White embedded embryo sections (1 μ m) using the method described previously (Ghosh *et al.* 1999, 2000a, 2000b, 2004, Dhara *et al.* 2001, Sengupta *et al.* 2003). Immunohistochemistry for all the three isoforms of NOS – eNOS, nNOS, and iNOS – were performed using antibodies (Cayman Chemical Co., MI, USA) raised in rabbits against recombinant human antigens. Dilutions of stocks of antibodies for immunohistochemistry were optimized. Final visualization was achieved using the ABC peroxidase kits (Vector Laboratories, Burlingame, CA, USA) and freshly prepared diaminobenzidine hydrochloride (Sigma) and hydrogen peroxide. For 1 μ m embryo sections, DAB enhancing solution (Vector Laboratories) was used to enhance the signal for better assessment and analysis. Specificity of the antibody binding and visualization were assessed by omitting primary antibodies, replacing primary antibody with unrelated immunoglobulins from the same species and other species, omitting secondary antibodies, and replacing labelled secondary antibody with unrelated labelled immunoglobulins from the same species and other species. Labelled and unlabelled immunoglobulins, non-immune sera and other supplies for immunohistochemistry were purchased from Vector Laboratories. Additional control assessment using preadsorbed immunoglobulins with recombinant control peptides (Cayman Chemical Co., MI, USA) was performed.

In situ hybridization

The *in situ* hybridization (ISH) protocol used in the present study was based on the method described earlier (Ghosh *et al.* 2000b) and using the antisense and sense probe templates for eNOS (271 bp), nNOS (330 bp), and iNOS (371 bp) from Cayman Chemical Co. Briefly, 5 μ m paraffin sections were dewaxed in xylene and hydrated through graded ethanol to PBS (pH 7.4). Proteinase K (10 μ g/ml in PBS; Amresco, Solon, OH, USA) digestion was performed at room temperature for 30 min. Following washes in PBS, sections were refixed in freshly prepared 4% neutral

buffered paraformaldehyde at 4°C for 20 min, and then were washed in PBS. Finally, dehydration was done with graded ethanol and air-dried.

On the other hand, single stranded sense and antisense RNA probes were transcribed from linearised templates using DIG RNA labelling kit (Boehringer-Mannheim, Mannheim, Germany) and using the protocol described by the manufacturer. Riboprobes (~5 ng/ μ l) were mixed with 40 μ l of hybridization mixture containing deionized formamide (50%, v/v), 50% (w/v) dextran sulphate (20%, v/v), 100X Denhardt mixture (1%, v/v), Tris (1 M, pH 8.0), sodium chloride, EDTA and yeast t-RNA (5 mg%) and were denatured at 80°C. The chemicals for hybridization were obtained from Sigma and Amresco (Solon, OH, USA). The riboprobes were applied to the pretreated and dried section, and hybridization was carried out for 18 h at 47°C in a sealed humidified chamber equilibrated with 50% formamide and 0.3 M NaCl. Post hybridization stringency washes were done in 2 $\times$ SSC and then 0.1 $\times$ SSC at 47°C. After washing in 2 $\times$ SSC at 37°C, the hybridized probes were detected by using DIG nucleic acid detection kit (Boehringer Mannheim, Mannheim, Germany) and using the protocol supplied by the manufacturer.

Microscopic assessment

At least three sections of each primary implantation site for each immunochemical staining and *in situ* hybridization were examined. Samples examined belong to either lacuna stage ($n = 6$) or villous placenta stage ($n = 9$). For assessment of staining in cells at implantation sites, semi-quantitative subjective scoring was done in a blinded manner by three investigators using a 4-scale system: 0 = nil; 1 = weak; 2 = moderate; 3 = strong (Ghosh *et al.* 1999, 2000b, 2004, Sengupta *et al.* 2003). It was assumed that these measurements reflect the concentrations of the experimental probes in different endometrial compartments. Immunohistochemistry and NADPH-diaphorase histochemistry of embryo samples were assessed subjectively.

Treatment groups

Female rhesus monkeys ($n = 45$) showing at least two consecutive cycles of normal length (26–32 days) were used in this part of the study and were randomly assigned to five groups, nine animals in each group. Female animals were cohabited with males from days 8–16 of their ovulatory cycles. Success in insemination was assessed by daily microscopic examination of vaginal smear during the mating period. On estimated day 6 after ovulation seven animals belonging to group 1 (control) were vaginally inserted with tampons (Tampex, Tambrands, Palmer, MA, USA) bearing a dialysis tube containing PBS (pH 7.4), while nine animals in groups two (4 mg/kg body weight) and three (6 mg/kg body weight) and seven animals in group four (8 mg/kg body weight) were vaginally inserted with tampons containing N^G-nitro-L-arginine methyl ester (L-NAME, Sigma), and eight animals in group

five (4 mg/kg body weight) with aminoguanidine hydrochloride (AG, Sigma), on post ovulation day 6 under light ketamine anaesthesia. The minimal amount of 4 mg NOS inhibitor per animal was arbitrarily adjusted using interspecies allometric scaling considering both allometric coefficient and exponent 0.9, and based on available human and mouse data for L-NAME from the literature (Chen *et al.* 2001, Mordenti 1986, Cotter *et al.* 2003, Morgan *et al.* 2003). Five animals did not show ovulation and were removed from the study. The description of a typical tampon containing the test agent or only PBS has been given earlier (Dhawan *et al.* 2000). Typically, a 3.0 cm × 1.2 cm tampon with 1.5 cm thread lead at the bottom was wrapped with a dialysis tube (length, 4.0 cm; diameter, 66 mm; 12000 kD cut-off) filled with buffer either alone or containing test agent. The tampons were removed on post ovulation day 12 of the treatment cycle under light ketamine anaesthesia. Pregnancy establishment was assessed from serum levels of oestrogen and progesterone, as well as, detection of mCG in circulation followed by uterine palpation as described earlier (Ghosh & Sengupta 1993, Ghosh *et al.* 1994, 1997). Pregnancy outcome was analysed using Fisher's exact ratio test, and area under the curve (AUC) for hormone levels during the days of treatment (days 6 to 12 after ovulation) for individual females was compared using ANOVA (Zar 1999).

Results

NOS in pre-implantation stage embryos

As shown in Fig. 1, pre-implantation stage monkey blastocysts showed marked NADPH-diaphorase activity, and expressed immunopositive iNOS, while morula stage embryos showed very little diaphorase activity and no immunopositive iNOS. Both NADPH-diaphorase activity and NOS immunopositivity were observed predominantly in the inner cell mass. However, no immunopositivity for eNOS and nNOS was seen in morulae or blastocysts.

NOS distribution at primary implantation site

Tables 1 and 2 show the median scores with ranges of immunohistochemical and *in situ* hybridization staining for eNOS, iNOS and nNOS in different compartments of conceptus and maternal endometrium at primary implantation sites during lacunar and villous placenta stages of implantation in the rhesus monkey. Fig. 2 shows representative microphotographs of NOS transcripts and proteins as detected by *in situ* hybridization and immunohistochemistry, respectively, in different compartments of conceptus and maternal endometrium during the early stages of blastocyst implantation and placentation.

Conceptus

During the lacunar stage of placentation, eNOS and iNOS expressions were observed in cytotrophoblast cells lining

the embryonic cavity (Table 1). The mRNA and protein expressions for all three isoforms of NOS were observed in the large trophoblast cells within the chorionic plate. As gestation advanced, cytotrophoblast cells lining villi, within cell columns, as well as in anchoring villi began expressing all the three isoforms of NOS with eNOS being the dominant isoform, while syncytiotrophoblast cells expressed NOS only marginally (Table 2; Fig. 2). Villous stromal cells showed expression of all the three isoforms of NOS.

Maternal endometrium

As shown in Table 1, during the lacunar stage of placentation, epithelial cells including plaque cells and decidual cells in the maternal endometrium at the primary implantation site generally expressed eNOS and iNOS. As gestation advanced, mRNAs for all three isoforms of NOS increased in epithelial and decidual cells, with no marked change in their protein expression profiles (Tables 1 and 2). The proteins and mRNAs for all three isoforms of NOS were generally very low in endothelial cells of maternal endometrial vasculature at the primary implantation site during both stages of placentation (Tables 1 and 2).

Pregnancy outcome following administration of NOS inhibitors

Table 3 shows the effect of vaginal administration of vehicle (group 1), L-NAME (groups 2–4) and AG (group 5) on pregnancy outcome in mated, ovulatory cycles of rhesus monkeys. In the control group (group 1), five out of seven animals (71%) became pregnant as revealed from steroid hormonal profiles and mCG in circulation. Pregnancy in two animals (C6 and C7) in this group (group 1, control) was allowed to continue, while pregnancy in three animals (C1, C2 and C3) in this group was terminated by the removal of implantation sites between cycle days 27 to 29. Pregnancy in group 2 was confirmed in two (22%) out of nine animals who were exposed to vaginal L-NAME (4 mg/kg body weight) during days 6 to 12 after ovulation, while there were four (44%) confirmed pregnancies out of nine animals in group 3 receiving vaginal L-NAME, 6 mg/kg body weight, and two (29%) confirmed pregnancies from seven animals in group 4 treated with vaginal L-NAME, 8 mg/kg body weight. On the other hand, two (25%) out of eight animals were pregnant in group 5 following treatment with AG during days 6 to 12 after ovulation. One animal from group 4 (L₈1) and one animal from group 5 (AG5) could not maintain their pregnancies and aborted on 47 and 19 days of gestation, respectively. Finally, twelve animals from all groups gave twelve normal babies (Table 3).

Despite an apparent tendency of inhibition in pregnancy establishment in L-NAME or AG treated animals as compared with control group animals, Fisher's exact probability test failed to detect any significant decrease in the pregnancy outcome in any group of animals treated with

Figure 1 Pre-implantation stage monkey embryos showing NADPH-diaphorase activity (A–D). Enzyme activity detected from formazan grains was low in morula (A), higher in zona-encased blastocyst (B), and maximum in cells of the inner cell mass (*) and trophoblast cells (t) of zona-free blastocyst (C), and very low in an early blastocyst in which β -NADPH was omitted (control) from the incubation medium (D). Immunohistochemical localization of iNOS in 1 μ m section of zona-free blastocyst was found mainly in cells of the inner cell mass (E). An adjacent section of the same blastocyst incubated in medium without primary antibody for iNOS showed no positive immunostaining (F). Bars = 20 μ m (A–D), 5 μ m (E, F).

NOS inhibitor as compared with group 1. Analysis of the AUC during days 6 to 12 after ovulation failed to reveal any significant change in the serum levels of oestradiol and progesterone between animals treated with vehicle and animals treated with inhibitor of NOS (either L-NAME or AG), as well as, between animals treated with L-NAME and animals treated with AG.

Discussion

In the present study, marked NADH-diaphorase activity and iNOS protein were observed in the inner cell mass of

implantation stage monkey blastocysts. There is no comparable study in any primate species in the literature, however, similar observations have been reported for pre-implantation stage rodent embryos (Nishikimi *et al.* 2001). While immunoprecipitation of a target protein essentially reveals its presence, but not its activity, and NADH-diaphorase activity *per se* is not sacrosanct of NOS activity in pre-implantation stage embryos, their marked concordance tends to substantiate the likelihood of NOS activity (Lopez & Gonzalez 2002). Although the physiological significance of this observation is not known, it is likely that NOS is involved in embryo development. There is

Table 1 Endothelial nitric oxide synthase (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) in conceptus and endometrium at the primary implantation site ($n = 6$) during the lacuna stage of placentation. Values are given as median scores with ranges in parentheses.

Cell type	eNOS		nNOS		iNOS	
	IHC	ISH	IHC	ISH	IHC	ISH
<i>Conceptus</i>						
Chorion	1 (0–2)	0 (0–2)	0 (0–1)	0 (0–1)	1 (0–2)	0 (0–1)
Trophoblast cells lining embryo cavity	2 (1–2)	1 (1–2)	1 (0–2)	0 (0–1)	2 (1–3)	1 (1–2)
Trophoblast cells lining lacunae	0 (0–1)	1 (1–2)	0 (0–1)	0 (0–1)	0 (0–1)	1 (0–1)
Trophoblast cells in chorionic plate	1 (0–2)	2 (1–3)	1 (0–1)	1 (1–2)	1 (0–2)	2 (1–3)
<i>Endometrium</i>						
Epithelium						
Luminal	2 (1–2)	1 (0–2)	1 (0–2)	0 (0–1)	2 (0–3)	1 (0–2)
Glandular	1 (0–2)	1 (0–2)	1 (0–1)	0 (0–2)	1 (0–1)	1 (0–1)
Plaque	1 (0–2)	2 (0–3)	1 (0–1)	1 (0–2)	2 (1–3)	2 (1–3)
Decidual cells	2 (0–3)	1 (0–2)	0 (0–1)	0 (0–2)	2 (0–3)	1 (1–2)
Fibroblast cells	0 (0–1)	1 (0–1)	0 (0–1)	0 (0–1)	0 (0–2)	1 (0–1)
Endothelial cells	0 (0–1)	0 (0–1)	0 (0–1)	0 (0–1)	0 (0–1)	0 (0–1)

IHC, immunohistochemistry, ISH, *in situ* hybridization.

evidence that an optimal level of NO is required for adequate and synchronous development of pre-implantation stage embryos in rodents (Chen *et al.* 2001, Tranguch *et al.* 2003). In a pilot study, it has also been observed that NO production in nidating human embryos is positively associated with the growth potential of a developing embryo (Battaglia *et al.* 2003).

In the present study, we noted that pre-implantation stage monkey blastocysts expressed immunopositive iNOS, while cytotrophoblast cells lining the embryonic cavity, villi, columns and anchoring villi during lacunar and villous stages of placentation in the rhesus monkey expressed both eNOS and iNOS. In the present study, generally a very low to moderately low level of nNOS was detected in most samples, although immunohistochemistry only gives a relative idea of semi-quantitative assessment at best. In previous reports, nNOS has been localized in trophoblast cells from human term placenta (Sanyal *et al.* 2000), but not in first trimester placental samples (Al-Hijji *et al.* 2003). In the present study, all the three isoforms of NOS were observed to a variable extent in extravillous trophoblast cells, non-polarized cytotrophoblast cells of column and villous stromal cells.

In previous studies also, eNOS protein has been located in the extravillous trophoblast cells of the cell columns of

anchoring villi in the human placenta (Ariel *et al.* 1998, Martin & Conrad 2000). In a recent study, on the other hand, neither eNOS nor iNOS immunopositivity was observed in extravillous cytotrophoblast cells in human placental samples collected between 8 and 19 weeks of gestation (Lyll *et al.* 1999). The authors suggested that such discrepancy might arise from the technical variations (for example, chemically fixation vs cryo fixation, polyclonal antibody vs monoclonal antibody) in performing immunohistochemistry. While we appreciate this possibility, it is notable that there is a good concordance in semi-quantitative assessments of relative levels between transcripts as detected by *in situ* hybridization and proteins as detected by immunohistochemistry in our study. Martin & Conrad (2000) also observed a discernible degree of transcripts for eNOS in extravillous trophoblast cells in human first trimester placenta samples. In the present study, markedly wide ranges of data were observed for several parameters in primary implantation sites primarily for sample (biological) variation rather than subjective (technical) variation, while uterine myometrial cells, similar to earlier reports in the human (Ramsay *et al.* 1996, Campa *et al.* 1998), showed only a low level staining in *in situ* hybridization and immunohistochemistry (data not shown). It is to be noted, in this connection, that the primary

Figure 2 *In situ* hybridization and immunohistochemistry of NOS isoforms in cells of conceptus and maternal endometrium in lacunar and villous stages of primary implantation sites collected from rhesus monkeys. Lacunar stage tissues showing eNOS (A), iNOS (B) and nNOS (C) mRNAs in cytotrophoblast cells (arrowhead) lining embryonic cavity and lacunae (l), chorionic plate (p), plaque acini (pl) and in glandular epithelial cells (gl). Immunohistochemical localization of eNOS (D), iNOS (E) and nNOS (F) in syncytiotrophoblast cells (arrows) lining lacunae (l), cytotrophoblast cells (ctb); marked immunostaining for iNOS (E) was found in migratory trophoblast cells (brackets) within the chorionic plate. Immunohistochemical localization of eNOS (G) in cytotrophoblast cells lining villi (arrowhead) and in cell columns (cc), syncytiotrophoblast cells lining cell columns (arrows), migratory extravillous trophoblast cells (brackets) adjoining blood vessel (bv) and gland epithelial cells (gl). *In situ* hybridization showing mRNAs for iNOS (H) in cytotrophoblast cells of columns (cc) and in decidual cells (d; l) surrounding spiral arterioles (*). *In situ* hybridization with the sense probe for iNOS did not show any detectable staining (J). No staining was found after immunohistochemistry for eNOS by omitting the primary antibody (K) and for iNOS after incubation in primary antibody immunosorbed with recombinant iNOS (L). Bars = 50 μ m (A–C, G–J), 10 μ m (D–F, K, L).

implantation site samples were collected from pregnant rhesus monkeys during very early stages of placentation, between days 12 and 17 of gestation, unlike all previous reported studies in primates including the human.

It is possible that trophoblast derived NO may play a critical role in preventing platelet adhesion to the trophoblast cell layer as well as inhibiting platelet aggregation within the intervillous space (Radomski *et al.* 1987). Because NO is a potent vasodilator, it might also contribute to the vasodilation of the maternal systemic circulation at the implantation site. Indeed, it has been earlier reported that NO produced by periarterial trophoblasts during early pregnancy in the guinea pig contribute to the dilation of the adjacent uteroplacental arteries (Nanaev *et al.* 1995). NO derived from these cells may also contribute to the local suppression of maternal immunity (Dembinska-Kiec *et al.* 1991, Belensky *et al.* 1993).

Interestingly, invasive extravillous trophoblast cells were found to express NOS, and to co-express VEGF and its receptors (Flt-1 and KDR) on days 12–16 of gestation in the rhesus monkey (Ghosh *et al.* 2000b, Ghosh & Sengupta 2004a). VEGF reportedly stimulates the release of NO via phosphorylation of Flt-1 in human first trimester trophoblast cells, and this action is attenuated by L-NAME in a concentration dependent manner, and NO in turn suggestively aids in trophoblast penetration into spiral arterioles (Ahmed *et al.* 1997) while down-regulating VEGF-stimulated proliferation of trophoblast cells (Cha *et al.* 2001). Whether NOS activity in extravillous trophoblast cells has any functional association with high expression of interleukin-1 (IL-1) and leukemia inhibitory factor (LIF) during the early stages of placentation (Sengupta *et al.* 2003) is not known, however, these cytokines are known to influence NO production in a number of tissue systems including endometrium and cultured trophoblast cells (Tamura *et al.* 1996, Chwalisz *et al.* 1996, Chwalisz & Garfield 2000,

Hambartsoumian *et al.* 2001). NO production by invasive trophoblast cells may induce matrix-degrading proteases, like matrix metalloproteinases 2 and 9, which were reduced by the addition of L-NAME to cells in culture (Novaro *et al.* 2001). It is likely that trophoblast cells which are epithelial in origin may require NO for inducing a switch from stationery to locomotory phenotype (Noiri *et al.* 1996).

Although there is no information available in the literature regarding the expression of NOS in maternal endometrium during the early stages of implantation and placentation in the primates, our observation corroborates well with the available data obtained from studies in the non-gravid premenopausal human uterus (Schmidt *et al.* 1992, Telfer *et al.* 1995, 1997, Tseng *et al.* 1996, Taguchi *et al.* 2000). In our findings, the expression of NOS by maternal endometrial epithelial cells of luminal surface, glands and plaque acini during early stages of implantation appears intriguing. A role for NO in the paracrine control of the uterine vascular bed is supported by studies in animals (Kimura *et al.* 1992, Van Buren *et al.* 1992). While locally synthesized NO by maternal endometrial plaque epithelium may play an important role in the control of the uterine vascular bed around the primary implantation site (Ziche *et al.* 1994), it is possible that NO along with other factors from surface and glandular epithelial cells mediate embryotropic action (Hornung *et al.* 1998, Ghosh & Sengupta 2004b).

In the present study, we also observed that decidual cells expressed NOS at a low level during lacunar stage, while its expression increased during the villous placental stage of implantation. It is possible that NOS expression in pregnancy associated decidual cells is associated with the process of decidualization. Chwalisz & Garfield (2000) reported that NO is involved in the process of decidualization, since NOS inhibitors reduced the extent

Table 2 Endothelial nitric oxide synthase (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) in conceptus and endometrium at the primary implantation site ($n = 9$) during the villous stage of placentation. Values are given as median scores with ranges in parentheses.

Cell type	eNOS		nNOS		iNOS	
	IHC	ISH	IHC	ISH	IHC	ISH
<i>Conceptus</i>						
Cytotrophoblast cells lining villi	2 (1–3)	1 (0–2)	0 (0–1)	0 (0–1)	2 (0–3)	0 (0–2)
Syncytiotrophoblast cells lining villi	1 (0–2)	0 (0–2)	0 (0–1)	0 (0–1)	0 (0–1)	0 (0–1)
Non-polarised cytotrophoblast cells in columns	2 (0–3)	2 (2–3)	1 (1–2)	2 (1–3)	2 (1–3)	2 (0–3)
Syncytiotrophoblast cells lining cytotrophoblast cell column	0 (0–1)	0 (0–1)	0 (0–1)	1 (0–2)	1 (0–2)	0 (0–1)
Trophoblast cells of cytotrophoblast shell	0 (0–1)	2 (1–3)	0 (0–1)	1 (1–2)	1 (0–2)	2 (1–3)
Extravillous trophoblast cells	2 (1–3)	2 (2–3)	1 (1–3)	1 (0–2)	2 (1–3)	2 (2–3)
Extraembryonic mesenchymal cells	2 (1–3)	2 (2–3)	1 (1–2)	2 (0–2)	2 (1–3)	2 (2–3)
<i>Endometrium</i>						
Epithelium						
Surface	2 (2–3)	1 (0–2)	1 (1–3)	1 (0–2)	2 (1–3)	1 (1–2)
Glandular	1 (1–3)	1 (0–1)	0 (0–2)	1 (0–2)	1 (1–2)	1 (1–2)
Plaque	1 (0–1)	3 (2–3)	0 (0–1)	2 (1–3)	2 (1–3)	3 (1–3)
Decidual cells	2 (1–3)	2 (0–2)	1 (0–1)	1 (1–3)	2 (1–3)	2 (1–3)
Fibroblast cells	1 (1–2)	0 (0–1)	0 (0–1)	0 (0–1)	0 (0–1)	0 (0–1)
Endothelial cells	1 (1–2)	0 (0–1)	0 (0–1)	0 (0–0)	0 (0–2)	0 (0–1)

Table 3 Pregnancy outcome in monkeys subjected to vaginal administration of vehicle (group 1), N^G-nitro-L-arginine methylester (4 mg/kg, group 2; 6 mg/kg, group 3; 8 mg/kg, group 4) and aminoguanidine (4 mg/kg, group 5).

Group	Animal number	Length of menstrual cycle			Serum mCG detection (days after ovulation)
		Pre-	Treatment	Post-	
1	C1	28	NA	NA	+, 12 ¹
	C2	29	NA	NA	+, 11 ¹
	C3	33	NA	NA	+, 13 ¹
	C4	30	29	29	ND
	C5	29	30	28	ND
	C6	30	NA	NA	+, 13 ^{2,1}
	C7	27	NA	NA	+, 12 ^{3,1}
2	L ₄ 1	26	26	24	ND
	L ₄ 2	30	27	29	ND
	L ₄ 3	27	28	27	ND
	L ₄ 4	29	28	37	ND
	L ₄ 5	27	19	61	ND
	L ₄ 6	32	NA	NA	+, 15 ^{2,2}
	L ₄ 7	28	NA	NA	+, 16 ^{3,2}
	L ₄ 8	31	32	32	ND
	L ₄ 9	26	25	29	ND
3	L ₆ 1	26	NA	NA	+, 15 ^{3,3}
	L ₆ 2	30	NA	NA	+, 17 ^{2,3}
	L ₆ 3	28	26	29	ND
	L ₆ 4	29	28	27	ND
	L ₆ 5	31	29	26	ND
	L ₆ 6	31	NA	NA	+, 18 ^{2,4}
	L ₆ 7	29	NA	NA	+, 16 ^{3,4}
	L ₆ 8	28	31	32	ND
	L ₆ 9	27	25	59	ND
4	L ₈ 1	28	NA	NA	+, 15 ^{4,1}
	L ₈ 2	28	25	29	ND
	L ₈ 3	28	28	32	ND
	L ₈ 4	30	28	43	ND
	L ₈ 5	27	29	31	ND
	L ₈ 6	29	NA	NA	+, 16 ^{2,5}
	L ₈ 7	28	NA	NA	+, 16 ^{3,5}
5	AG1	27	24	33	ND
	AG2	30	22	29	ND
	AG3	26	42	15	ND
	AG4	27	NA	NA	+, 16 ^{2,6}
	AG5	26	NA	NA	+, 13 ^{4,2}
	AG6	27	55	30	ND
	AG7	26	NA	NA	+, 19 ^{3,6}
	AG8	28	27	27	ND

of decidualization in rats. It is also possible that the expression of NOS by stromal decidual cells may influence the vascular responses during implantation (Rogers *et al.* 1992, Ziche *et al.* 1994, Okada *et al.* 2001). Several cytokines including interleukin-1 (IL-1) and leukemia inhibitory factor (LIF) in implantation stage endometrium (Sengupta *et al.* 2003) induce an optimal amount of NO production in progesterone dominated endometrium (Franchi *et al.* 1998, Okada *et al.* 2001, Novaro *et al.* 2002); NO, in turn, stimulates the production of matrix metalloproteinases resulting in remodeling of extracellular matrix cooperating the process of implantation (Chwalisz *et al.* 1996, Tamura *et al.* 1996). NO may also potentiate progesterone mediated smooth muscle relaxation during pregnancy (Weiner *et al.* 1994, Chwalisz *et al.* 1996).

In our study, we observed a reduction in the percentage of pregnancy by the administration of a non-specific inhibitor of NOS, L-NAME (22% at 4 mg, 44% at 6 mg, and 29% at 8 mg per kg body weight doses), as well as, a selective but not very specific inhibitor of iNOS, aminoguanidine (AG, 25%) (Joly *et al.* 1994) as compared with control treated (71%) animals. Although these differences are statistically not significant, these changes are noteworthy. In fact, a proportion test analysis of the present set of data for different groups following 1000 replicate bootstrapping (Efron *et al.* 1996) reveals a very high level of significance ($P < 0.001$) in inhibition of pregnancy as the posterior probability of peri-implantation stage administration of NOS inhibitors. The observed decrease in the rate of successful pregnancy in the present study when viewed with a background, and an increasing bulk, of evidence suggesting a potential involvement of NO in the cytokine network (Schwentker *et al.* 2002), in the regulation of gene expression through modulation of transcription factors (Sen & Packer 1996) and as a common downstream pathway for protein kinase C (PKC) and tyrosine kinases (Xuan *et al.* 1999) indeed supports the notion that timed inhibition of NOS activity during peri-implantation stage is likely to result in an interception in the establishment of pregnancy.

In the present study, the dose-response of L-NAME tested at higher doses of 6 mg and 8 mg per animal failed to increase the contraceptive efficacy of the drug. While the cause-effect relationship is only speculative at this point in time, it appears from our study with the monkey model that local administration of NOS inhibitor for targeted anti-implantation strategy in the primate results in a complex scenario. The pharmacokinetics of NOS inhibitor bioavailability at the local tissue bed in the given experimental model is not known, but the relatively low PO_2 values around the time of blastocyst implantation and in the placenta during the first trimester of pregnancy (Yedwab *et al.* 1976, Rodesch *et al.* 1992, Graham *et al.* 2000) may function to modulate NO bioavailability. It is known that low ambient pO_2 , through enhanced binding to oxygenated hemoglobin, participates in physiological microvascular regulation, and hypoxemia enhances NO concentration while hyperoxemia results in accelerated NO removal (Heyman *et al.* 1999). We did not observe any marked change either in systemic blood pressure (Takiuti *et al.* 1999) or in sleep cycle pattern (Sippel *et al.* 1999) or on food intake pattern (Vozzo *et al.* 1999) in animals during the period of vaginal administration of NOS inhibitor (data not shown). We believe that locally administered NOS inhibitor was operative at the local level based on the fact that all the babies in the NOS inhibitor treated groups had significantly ($P < 0.05$) lower body weights as compared with babies from pregnant females in the control group and in our monkey colony in general. It is thus possible that local treatment with NOS inhibitors affect the NO system in fetal and maternal compartments resulting in retardation of embryonic growth without

affecting ovarian function as no change in the levels of ovarian steroidal hormones was observed in NOS inhibitor treated animals. The embryotropic and anti-apoptotic actions of NO are now well documented in several biological models (Gouge *et al.* 1998, Dash *et al.* 2003, Teodoro & O'Farrell 2003, Tranguch *et al.* 2003).

Collectively, it appears from the present study that there exists a spatio-temporal pattern of NOS-expression in a cell-type specific manner in the embryo and maternal endometrium during implantation and placentation, and that NO plays an important role in the process of successful establishment of pregnancy in the rhesus monkey.

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