The expression of inducible nitric oxide synthase in the human fallopian tube during the menstrual cycle and in ectopic pregnancy

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Objective: To investigate the production of inducible nitric oxide synthase (iNOS) in the fallopian tube (FT) during the menstrual cycle and whether epithelia from FTs bearing an ectopic pregnancy differ from healthy tubes in iNOS expression.
Design: Prospective study.
Setting: Academic unit of reproductive and developmental medicine.
Patient(s): Fallopian tubes from the different stages of the menstrual cycle (n = 12), FTs bearing an ectopic pregnancy (n = 15), and FTs from pseudopregnant women (n = 6) were collected.
Intervention(s): In the pseudopregnant group, patients were injected with hCG in the days leading up to hysterectomy. Samples were processed for immunohistochemistry staining and quantitative reverse transcriptase polymerase chain reaction.
Main Outcome Measure(s): To compare iNOS protein and messenger RNA expression between the different groups.
Result(s): This is the first report on cyclicity in iNOS production by human fallopian tube during the menstrual cycle. The intensity of expression of iNOS was higher in the ectopic pregnancy group compared with the pseudopregnant group ($P < 0.05$).
Conclusion(s): The cyclicity in iNOS expression by the tube suggests its involvement in fertilization and early embryonic development. Pathologic generation of nitric oxide through increase iNOS production may decrease tubal ciliary beats and smooth muscle contractions and thus affect embryo transport, which may consequently result in ectopic pregnancy. (Fertil Steril® 2010;94:833–40. ©2010 by American Society for Reproductive Medicine.)

Key Words: Early embryonic development, ectopic pregnancy, fallopian tube, menstrual cycle, nitric oxide

Nitric oxide (NO) is a free radical with a half-life of only few seconds and is synthesized from L-arginine by the action of NO synthase (NOS), an enzyme existing in three isoforms (1). The three isoforms of NOS are products of separate genes that share 50–60% amino acid homology (2). Neuronal NOS (nNOS or NOS1) and endothelial NOS (eNOS or NOS3), are responsible for the continuous basal release of NO and both require calcium/calmodulin for activation (3, 4). An increase in intracellular calcium triggers a cascade of events leading to eNOS activation and NO synthesis. A third isoform is an inducible calcium-independent enzyme (iNOS or NOS2) that is expressed only in response to inflammatory cytokines and lipopolysaccharides (5, 6). In contrast to eNOS, iNOS contains calmodulin tightly bound to each subunit of the enzyme, and therefore activation of iNOS does not require an increase in intracellular calcium and results in the permanent activation of the enzyme (7).

An ectopic pregnancy is any pregnancy that develops after implantation of the blastocyst outside the endometrial lining of the uterine cavity. Ectopic pregnancy is associated with significant maternal morbidity and mortality, and its incidence is increasing worldwide (8). It is the leading cause of pregnancy-related death in the first trimester, accounting for 9% of all pregnancy-related deaths (9, 10). The incidence of ectopic pregnancy in United Kingdom is 11.1 per 1000 pregnancies, and nearly 32,000 ectopic pregnancies were diagnosed in the United Kingdom within a recent three year period (1997–1999) (11). More than 95% of ectopic

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pregnancies occur in the fallopian tubes (tubal pregnancy), mainly in the ampullary region (12).

Effective tubal transport of sperm, ova, and embryo is essential for fertilization, early embryo development, and successful intrauterine pregnancy. Transportation within the tube is achieved by a complex interaction between muscle contractions, ciliary activity, and the flow of tubal secretions (13). Current knowledge about ciliary activity and its physiologic regulation is limited. Altered ciliary activity and/or ineffective tubal contractions may result in infertility or tubal implantation and the development of ectopic pregnancy (13).

The production of NO by the human fallopian tube has been previously reported (14), and a potent relaxing effect on human tubal smooth muscle was documented (14, 15). We therefore speculated that pathologic alteration in the expression of NO by fallopian tube epithelial cells may result in pathologic relaxation of the tubal smooth muscles, failure of propulsion of the early embryo along the fallopian tube, and the development of ectopic pregnancy. Our study investigated the expression of inducible NOS, which is related to inflammation and infection, in the different regions of the human fallopian tube during the different stages of the menstrual cycle, and whether epithelium from fallopian tubes bearing an ectopic pregnancy differed from healthy tubes in expression of inducible NOS.

A better understanding of the mechanism by which an embryo implants in the tubal epithelium, rather than in the uterus, may lead to improved methods for early diagnosis of ectopic pregnancy or approaches for avoidance of ectopic implantation in women with a previous history of ectopic pregnancy.

MATERIAL AND METHODS

The study was approved by the South Sheffield Ethics Committee and informed written consent was obtained before the collection of tissue samples. All specimens were collected at the Jessop Wing, Royal Hallamshire Hospital in Sheffield, United Kingdom.

Case Group

Tissue from 15 fallopian tubes was obtained from women with an ectopic pregnancy for whom salpingectomy was performed on clinical management grounds. All participants in this group conceived spontaneously and were not taking exogenous progesterone.

Control Group

Pseudopregnancy group Because it is not possible to collect fallopian tube tissue from women carrying a healthy pregnancy, we studied tissue collected at the time of hysterectomy. Women were asked to have treatment with hCG in the days leading to hysterectomy. This regimen produces a state of pseudopregnancy, is harmless, and has been previously used within U.K. research studies (16, 17). Six fallopian tubes were obtained from women who were undergoing routine total abdominal hysterectomy (TAH) for benign disease not affecting the fallopian tubes. These patients were pretreated with subcutaneous hCG (5000 IU) every 3 days beginning in the midluteal phase for at least 12 days before the TAH. The three included patients had had nine, six, and 10 doses, respectively. The menses were delayed in the patients by 24, 15, and 30 days, respectively. To confirm the pseudopregnancy status, an endometrial biopsy sample and a blood sample were collected at the time of hysterectomy to examine decidualization of the endometrium and progesterone and the hCG concentration. All women who donated fallopian tubes had regular menstrual cycles and were of proven fertility with no evidence of tubal disease.

Menstrual cycle group Twenty-four fallopian tubes were obtained from 12 cyclic women who were undergoing routine TAH for benign disease not affecting the fallopian tubes. All women who donated fallopian tubes had regular menstrual cycles, were of proven fertility with no evidence of tubal disease, and were not taking exogenous hormones.

Sampling and Processing

For the case group, the fallopian tubes were excised at least 1 cm from the implantation site to avoid collecting any embryonic or trophoblastic tissue and to assure the integrity of tubal morphology and function. The ampullary and isthmic regions of the excised tubes from the three groups were identified and a small section immediately cut from each region using RNAse-free equipment (baked at 200°C for 4 hours). These samples were then divided into three equal pieces, with one piece being immediately fixed in 10% buffered formalin for immunohistochemistry and the other parts in 5 mL of RNAlater solution (Ambion, Warrington, United Kingdom) for the reverse transcriptase polymerase chain reaction (RT-PCR). All the tissues used in the RT-PCR were snap-frozen in RNAlater solution (Ambion) and stored at –80°C until fixed in 10% buffered formalin to preserve the RNA stability.

Antibodies

A polyclonal rabbit antibody to detect inducible NOS was obtained from Affinity Bioreagents (Loughborough, United Kingdom). According to the manufacturer, this antibody preparation has been shown to detect iNOS in human samples using Western blot and immunohistochemical techniques. Blocking peptides for the iNOS polyclonal antibody was obtained from Autogen Bioclear (Wiltshire, United Kingdom).

Immunohistochemistry An avidin-biotin horseradish peroxidase technique was used to localize the expression of iNOS in the different samples following the protocol described previously (18). Briefly, sections were dewaxed, dehydrated in alcohol, and treated with 2% (vol/vol) hydrogen peroxide for 20 minutes in methanol to block endogenous peroxidase (19). The sections used were pretreated in an 850-W domestic microwave oven in 0.01 M citrate buffer for 6 minutes. The sections were incubated for 30 minutes with normal goat serum (Vector Laboratories, Burlingham, CA) then