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Altered Endometrial Expression of Endothelial Nitric oxide Synthase (eNOS) in women with Unexplained Recurrent Miscarriage and Infertility

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Abstract

Background—Endothelial nitric oxide synthase (eNOS) has diverse roles in the female reproductive system including a role in blastocyst implantation. Aberrant expression of eNOS could therefore be significant in the pathogenesis of disorders of implantation

Materials and Methods—eNOS protein and mRNA levels in the endometrium of women with recurrent miscarriages, unexplained infertility, and a control group was determined by compartmental quantitative immunohistochemistry and real time RT-PCR

Results—eNOS was immunolocalized to all layers of the endometrium and the vascular endothelium. eNOS protein expression was higher in glandular epithelium (P=0.004) and luminal epithelium (P=0.002) but not vascular endothelium and stroma (P=0.14) in women with recurrent miscarriage. Similarly, in women with unexplained infertility eNOS expression was significantly higher (P<0.03) in luminal epithelium but not in any other compartments compared with the control group. The levels of mRNA expression as determined by real time RT-PCR confirmed the protein data demonstrating higher eNOS mRNA expression In the endometrium of women with recurrent miscarriage and unexplained infertility compared with controls

Conclusion—Increased expression of eNOS in glandular and luminal epithelium of the endometrium in women with recurrent miscarriages and unexplained infertility suggests a detrimental effect of excess nitric oxide in endometrial receptivity and implantation

Keywords

recurrent miscarriage; unexplained infertility; eNOS; endometrium; Immunohistochemistry; Real-Time RT PCR

Introduction

Recurrent miscarriage (RM) is described as at least three consecutive spontaneous pregnancy losses in the first trimester and compromises 3% of couples (Regan and Rai 2000; Taylor 2003). Genetic and developmental abnormalities are the major causes of RM, however immune, endocrine, and endometrial anatomical or non-anatomical factors as well

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as thrombophilias can lead to RM. In about 50% of cases, the cause of miscarriage remains unknown (Li *et al.* 2002a; Li *et al.* 2002b). Unexplained infertility (UI) affects 15% of infertile couples and is defined as when all the tests of a basic infertility evaluation including semen analysis, hysterosalpingogram, ovarian reserve testing, pelvic ultrasound, and possibly laparoscopic evaluation of the pelvis are within normal limits (Hatasaka 2011; Smith *et al.* 2003). Recent data has shown that UI and RM are distinctly different diagnoses and UI is not due to recurrent pre-clinical pregnancy loss (Koot *et al.* 2011), although these two conditions might share common endometrial function defects.

Nitric oxide (NO) is a vasodilator synthesized from L-arginine through the action of nitric oxide synthase (NOS). The three isoforms of NOS which catalyze the formation of NO are expressed in the human endometrium although eNOS is the predominant form (Khorram *et al.* 1999). The endometrial expression of eNOS is cyclic with peak expression during the window of implantation in humans (Khorram *et al.* 1999; Ota *et al.* 1998), and rodents (Purcell *et al.* 1999). Both estrogen and progesterone (Han *et al.* 2005; Khorram and Han 2009; Zervou *et al.* 1999) regulate the expression of eNOS in the human endometrium (Khorram *et al.* 1999). Nitric oxide by virtue of its properties as a potent vasodilator (Palmer *et al.* 1987), a myometrial smooth muscle relaxant (Buxton 2004; Norman *et al.* 1997), and its participation in signal transduction pathways (Thomas *et al.* 2008) might play a significant role in establishment and maintenance of pregnancy. By virtue of these properties of NO we postulated that aberrant endometrial expression of eNOS as in endometriosis (Dong *et al.* 2001; Khorram and Lessey 2002; Ota *et al.* 1998; Wu *et al.* 2003) and adenomyosis (Ota *et al.* 1998) could occur in patients with UI and RM. Since oxidative stress plays an important role at least in idiopathic recurrent pregnancy loss (Gupta *et al.* 2007), and NO in high concentrations can induce nitrosative stress (Agarwal *et al.* 2008) we hypothesized that increased endometrial eNOS expression and thereby NO generation in patients with UI and RM could impair endometrial function by either inducing cellular apoptosis (Wang *et al.* 2010), or through nitrosylation of key endometrial proteins (Gu *et al.* 2010; Weiner *et al.* 2009) impair their physiological function.

Materials and Methods

Sample collection and preparation of sections

The protocol for this study was approved by the Human Subjects Committee at Shahid Beheshti Medical University. Endometrial biopsies were obtained from 3 groups of women using a pipelle curette 7–9 days post ovulation as determined by serial ultrasound scans. The RM group (N=10) consisted of women with a mean age of 32.8 with a mean of 4.7 consecutive pregnancy losses. Women with secondary miscarriages or less than three miscarriages were excluded. Evaluation of RM group including karyotype analysis, antiphospholipid antibody and thrombophilia testing were all within normal range. Endometrial cavity as assessed by hysterosalpingogram was normal in RM patients. Women with unexplained infertility (N=10) consisted of individuals with a mean age of 29.8 years who were unable to conceive for more than 2 years with a normal basic infertility evaluation. This evaluation consisted of endocrine tests (TSH, cycle day 3 FSH and estradiol levels, prolactin, progesterone levels greater than 10ng/ml in mid luteal phase); anatomical tests (hysterosalpingography and pelvic ultrasonography), and semen analysis (WHO criteria). The control group (N=10) consisted of women with a mean age of 36.1 years who presented for tubal sterilization. Women in this group had normal menstrual cycles (26–33 days), had a mean parity of 1.4 and had no prior history of pregnancy losses and no prior use of assisted reproductive techniques for conception. Endometrial biopsy specimens were divided into 3 portions; one piece was placed in 4% paraformaldehyde tissue fixative for 24h and then switched to 70% ethanol for later processing. Another piece was placed in RNA Later preservative and stored at –80°C, and one section was fixed for histological dating of

the endometrium using the criteria of Noyes et al. (Noyes *et al.* 1975). Human placental tissue was used as a positive control (Bhuiyan *et al.* 2006).

Quantitative Immunohistochemistry

Endometrial specimens were cut into 6 μ m sections using a Cryocut and placed on poly l-lysine coated slides and stored at -70°C for immunostaining. A monoclonal mouse antihuman antibody [6H2] (ABcam Company, UK, Cat.NO:91205) was used to detect the intensity and distribution of eNOS immunostaining using standard immunohistochemical protocol. Briefly after rinsing the slides with buffer (0.1 M of phosphate-buffered saline with a pH of 7.4), endogenous peroxidases were quenched by incubation in 0.3% H_2O_2 in methanol for 15 minutes. Repeated rinses with 0.05% bovine serum albumin in phosphate-buffered saline were performed followed by antigen retrieval using trypsin. 1.5% normal goat serum (DAKO Co., Denmark) was then added to the slides in humidified chambers for 20 minutes at room temperature to prevent nonspecific binding of antibody. The primary antibody against eNOS (1:100) was added to slides and incubated at 37°C for 1h. Slides were then rinsed three times with wash buffer, followed by incubation of sections with the secondary antibody, a rabbit antimouse IgG H&L (ABcam Company, Cat.NO: ab6728, UK) diluted 1:1000 in PBS. Incubation with the secondary antibody was performed for 1h at 37°C in an incubator. Slides were then exposed to 3, 3'-diaminobenzidine in H_2O_2 (DAKO co, Denmark) was for 15 minutes. Thereafter, the sections were counterstained with hematoxylin and mounted. In case of negative controls similar method was used but phosphate-buffered saline replaced the primary antibody. Human full term placental tissue served as an external positive control. Staining intensity of sections was determined by Image Pro Plus software by a blinded reviewer (TN) using previously described methods (Khorram *et al.* 2007). 6 different areas of the sections were analyzed at a magnification of 40x and the mean was used for statistical analysis. The results were expressed as % integrated Optical Density (%IOD).

Real-Time Reverse Transcriptase–Polymerase Chain Reaction

RNA was isolated using the High Pure RNA Isolation Kit (Roche applied science). Ribonucleic acid was DNase treated and quantitated by measurement of absorbance in a NanoDrop spectrophotometer. One microgram of total RNA was reverse transcribed into single stranded complementary DNA (cDNA) with use of the Omniscript Reverse Transcription kit (GeneON, Germany) at 37°C for 60 minutes in a total volume of 20 μL . The polymerase chain reaction (PCR) mix consisted of 1 μL of 10-fold diluted cDNA, qPCR MasterMix Plus for SYBR green I reagent (GeneON, Germany) and optimized forward and reverse gene-specific primers (300 nmol/L each). Real-time PCR reactions in triplicate were run in 96-well plates with use of an Mx3000P real-time PCR system (Stratagene, Santa Clara, CA). Reactions were started by activation of DNA polymerase at 95°C for 10 minutes followed by 40 PCR cycles of denaturing at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. The internal control used was human HPRT (de Kok *et al.* 2005). Data was analyzed to select a threshold level of fluorescence that was in the linear phase of the PCR product accumulation (the threshold cycle [CT]) for that reaction. The CT value for HPRT was subtracted from the CT value of eNOS gene to obtain a delta CT (DCT) value. The relative fold change for each gene was calculated with use of the $\Delta\Delta\text{CT}$ method (Livak and Schmittgen 2001).

Data analysis

Results were analyzed by ANOVA comparing %IOD for the immunohistochemical data and fold change in case of Real time RT-PCR using SPSS software. Post hoc analysis was done using the Student- Newman- Keuls test. $P < .05$ was considered statistically significant.

Results

The demographics of the three groups of women studied are shown in Table 1. Women in the UI group were significantly younger than the controls. There were no differences in BMI among the three groups. Four subjects in the control group, and two in the RM and UI groups were light smokers (<3 cigarettes/day). None of the subjects had chronic medical illnesses. Laparoscopic evaluation in all three groups showed a normal pelvis.

As previously demonstrated eNOS was expressed in all layers of the endometrium (Fig.1). Representative sections demonstrating eNOS protein staining in glandular (panels A–C) and luminal epithelium (panels D–F) (most intense staining) for the three studied groups are shown in this figure. Weak staining of eNOS was detected in the stromal layer (panel H), and vascular endothelium (panel G). eNOS expression was significantly higher in glandular epithelium of patients with RM (panel B) and UI (panel C) compared with control (panel A). A similar pattern was found in the luminal epithelium with greater expression of eNOS in patients with RM (panel E) and UI (panel F) compared with controls (Panel D). Negative and positive controls for eNOS are shown in panels I and J respectively. A summary of image analysis for eNOS expression in different layers of the endometrium are shown in Fig. 2. In women with recurrent miscarriage glandular ($P=0.004$) and luminal epithelial ($P=0.002$) expression of eNOS are greater compared with controls, whereas stromal ($P=0.14$) and vascular endothelial expression of eNOS was not significantly different in RM and UI women as compared with controls. In women with unexplained infertility the expression of eNOS was only higher in luminal epithelium compared with controls with no significant differences in other layers.

The expression of eNOS mRNA in the endometrium of women with RM and UI was significantly ($P<0.05$) higher compared with controls (Fig. 3) confirming the protein data.

Discussion

Our data confirms our prior reports on endometrial eNOS immunolocalization (Khorram *et al.* 1999), and demonstrates a differential site specific alteration in expression of eNOS protein in women with UI and RM compared with controls during the window of implantation. The most prominent changes were found in the luminal epithelium with greater expression of eNOS protein in both the RM and UI groups in this area. The expression of eNOS mRNA in endometrium of women with UI and RM was significantly higher compared with controls thus confirming the protein data. The luminal eNOS protein changes in both RM and UI groups support the importance of this enzyme for implantation and its dysregulation as a possible cause of implantation failures in these patients.

The significance of NO in the implantation process has been demonstrated in animal studies in which pharmacological blockers of NOS impair implantation. Novaro *et al.* demonstrated that the expression of NOS and PGE and PGF increase 1 day before implantation and suppression of NOS by L-NAME decreased production of PGE and PGF2 on the day of implantation in rats (Novaro *et al.* 1996). Biswas *et al.* (Biswas *et al.* 1998) injected L-NAME into the rat uterine horn and demonstrated an inhibition of implantation, and similar results were obtained by Duran-Reyes *et al.* using a different NOS inhibitor (Duran-Reyes *et al.* 1999). In contrast to these studies knockout mice for various isoforms of NOS do not show reduced litter size (Huang *et al.* 1993; Huang *et al.* 1995; MacMicking *et al.* 1995), indicating the importance of multiple pathways and redundancy in the implantation process. Since implantation failure secondary to endometrial factors may be common to UI (Koot *et al.* 2011) and RM (Li *et al.* 2002b) we sought to determine if aberrant endometrial expression of eNOS could be associated with these two conditions. The common finding of

luminal increase in the expression of eNOS protein in both RM and UI patients suggest that luminal eNOS is important in the pathogenesis of these disorders. Excess NO could impair implantation through several mechanisms. NO has been shown to induce endometrial epithelial apoptosis (Castro *et al.* 2002; Johnson *et al.* 2004; Li *et al.* 2001), and increased eNOS expression and therefore localized excess NO production at the luminal surface could induce epithelial apoptosis and implantation failure. Vatansever et al also reported increased eNOS immunoreactivity in the endometrium of unexplained infertility patients, although this was associated with lower number of apoptotic cells (Vatansever *et al.* 2005). A second mechanism by which NO might impair implantation is through localized nitrosative stress. Nitric oxide by virtue of its unpaired electron is a highly reactive free radical which in excess can damage protein, carbohydrates, nucleotides and lipids (Agarwal *et al.* 2008). Based on our data we propose that excess eNOS expression in luminal epithelium of patients with RM and UI can create local oxidative stress which could impair implantation, similar to other inflammatory gynecologic conditions such as endometriosis (Dong *et al.* 2001; Khorram and Lessey 2002; Ota *et al.* 1998; Wu *et al.* 2003), adenomyosis (Ota *et al.* 1998), and adhesions (Saed and Diamond 2004). Exogenous factors such as cigarette smoking which has been associated with recurrent miscarriages (Cramer and Wise 2000) can also induce nitrosative stress through direct endometrial cell stimulation of eNOS expression, an effect which can be blocked by anti-oxidants such as ascorbic acid (Khorram *et al.* 2010). Similarly ascorbate has recently been shown to activate eNOS activity by rapid modulation of its phosphorylation status (Ladurner et al., 2012).

Several studies have attempted to find a genetic link for RM, and in so doing have examined polymorphism of eNOS gene in different ethnic groups. A recent meta-analysis of these studies showed a significant association in eNOS (Glu298Asp) polymorphism and RM (Su *et al.* 2011). These genetic abnormalities could lead to reduced NO production and impaired endometrial function. Our data did not show any differences in endothelial microvascular eNOS levels in RM and UI patients, suggesting that localized endometrial blood flow mediated by the NO pathway during the implantation window is not a significant factor in the pathogenesis of these disorders, and other blood flow regulating factors not examined in this study may be of greater importance. Although weak expression of eNOS in vascular endothelium as seen in our endometrial samples could be a factor which can lead to infertility and miscarriage, these expression levels were not significantly different compared to the control group. This suggests that dysregulated expression of eNOS in non-endothelial sites plays a more significant role in the pathophysiology of recurrent miscarriages and unexplained infertility.

In conclusion our data demonstrates an over expression of eNOS protein and mRNA in the endometrium of RM and UI. This aberrant pattern of eNOS protein and mRNA expression is similar to other inflammatory gynecologic conditions such as endometriosis and adenomyosis. Although eNOS expression in some amounts is essential for implantation, excess eNOS expression and therefore excess generation of NO in the endometrium of patients with RM and UI is deleterious and could induce nitrosative stress which could lead to implantation failures or failure of early pregnancy maintenance.

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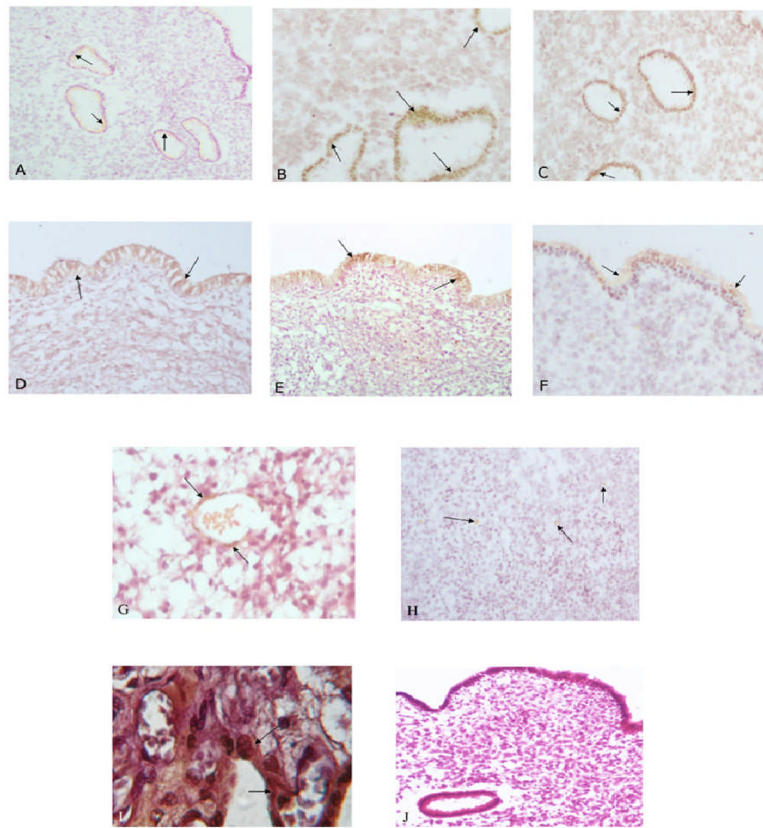


Figure 1. Shows eNOS staining in different endometrial compartments of the study population. Panel A shows immunostaining of eNOS in glandular epithelium of a control subject, RM patient (panel B), UI patient (panel C). Panel D shows eNOS staining in luminal epithelium of a control subject (panel D), RM patient (panel E), and UI patient (panel F). Panel G shows eNOS staining in vascular endothelium, and stroma (Panel H) of a control subject. Panel I is the negative control and panel J is positive control showing eNOS staining in a human placental section. Magnification is 40x.

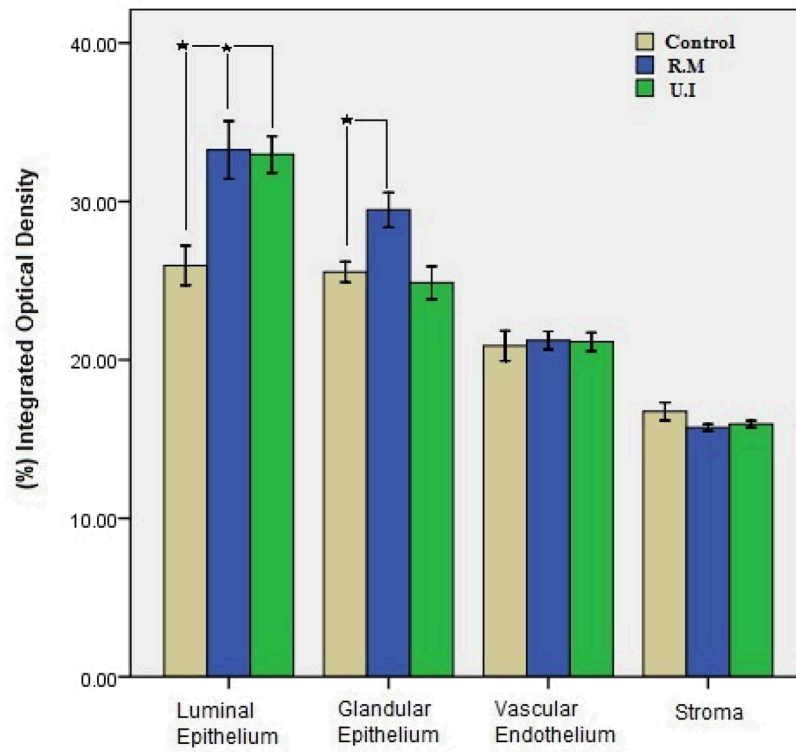


Figure 2. Bar plot summarizing eNOS protein quantification by image analysis in 4 compartments of the endometrium in control, recurrent miscarriage (RM) and unexplained infertility (UI) groups. * Statistical Significance.

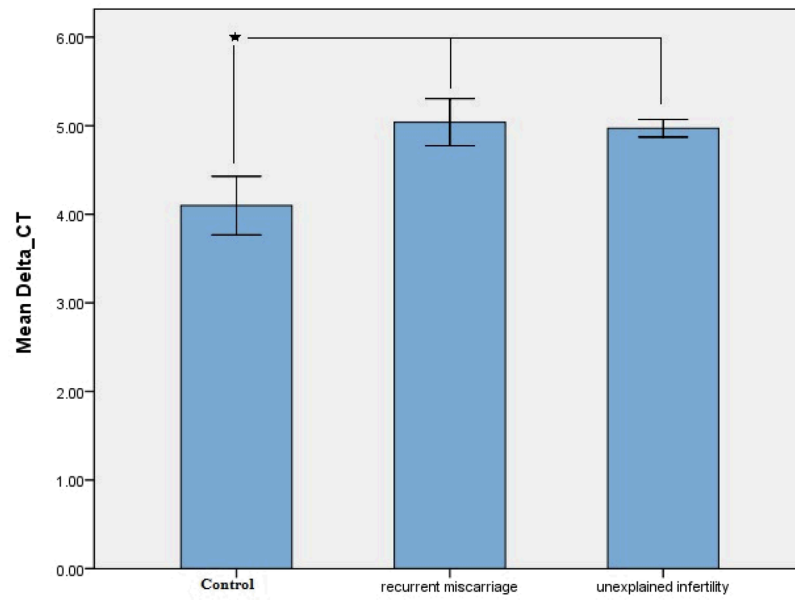


Figure 3. Shows eNOS mRNA levels in whole endometrial tissue homogenates in control, recurrent miscarriage and unexplained infertility groups.

Table 1

Shows the demographics of the study populations. Values are mean±SEM.

	<u>Age</u>	<u>BMI</u>	<u>Live Birth</u>	<u>Miscarriage</u>
Control	36.1± 1.1	26.1± 1.6	1.4± 0.2	0
Recurrent Miscarriage	32.8± 1.9	23.9± 1	0	4.7± 0.7
Unexplained Infertility	29.8±0.8	25± 1	0	0