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ARTICLE

Altered endometrial expression of endothelial nitric oxide synthase in women with unexplained recurrent miscarriage and infertility

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Tohid Najafi, MSc Embryology and Anatomical Science, was born in Iran and graduated from Shahid Beheshti Medical University in 2011. His major research fields are reproductive biology, specifically expression of nitric oxide synthase in normal and infertile endometrial tissues. He has also published articles about other aspects of the reproductive tract, for example regarding IVF and ultrastructure of spermatozoa. He also worked as an embryologist in infertility treatment centres in Iran. Tohid now started to pursue his PhD in Reproductive Endocrinology at Wayne State University of Michigan, USA Iran.

Abstract 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. In this study, eNOS protein and mRNA levels in the endometrium of women with recurrent miscarriages, unexplained infertility and a control group were determined by compartmental quantitative immunohistochemistry and real-time reverse-transcription PCR. eNOS was found to be immunolocalized to all layers of the endometrium and vascular endothelium. eNOS protein was higher in glandular epithelium ($P = 0.004$) and luminal epithelium ($P = 0.002$), but not vascular endothelium and stroma, in women with recurrent miscarriage. Similarly, in women with unexplained infertility, eNOS was significantly higher ($P < 0.03$) in luminal epithelium but not in any other compartments compared with the control group. The levels of mRNA confirmed the protein data, demonstrating higher eNOS mRNA in the endometrium of women with recurrent miscarriage and unexplained infertility compared with controls. In 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: endometrium, eNOS, immunohistochemistry, real-time RT-PCR, recurrent miscarriage, unexplained infertility

Introduction

Recurrent miscarriage 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 recurrent miscarriage; however, immune, endocrine and endometrial anatomical or non-anatomical factors as well as thrombophilias can lead to recurrent miscarriage. In about 50% of cases, the cause of miscarriage remains unknown (Li et al., 2002a,b). Unexplained infertility affects 15% of infertile couples and is defined as when

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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 have shown that unexplained infertility and recurrent miscarriage are distinctly different diagnoses and that unexplained infertility is not due to recurrent preclinical 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). Three isoforms of NOS 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 oestrogen 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). NO, by virtue of its properties as a potent vasodilator (Palmer et al., 1987) and 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, this study 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 unexplained infertility and recurrent miscarriage. 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), it was also postulated that increased endometrial eNOS expression and thereby NO generation in patients with unexplained infertility and recurrent miscarriage 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), impairing 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 (84th Session, 5/2010). Endometrial biopsies were obtained from three groups of women using a pipelle curette 7–9 days post ovulation as determined by serial ultrasound scans. The recurrent miscarriage group ($n = 10$) consisted of women with a mean age of 32.8 years and a mean of 4.7 consecutive pregnancy losses. Women with secondary miscarriages or less than three miscarriages were excluded. Evaluation of recurrent miscarriage group including karyotype analysis, antiphospholipid antibody and thrombophilia testing were all within normal range. Endometrial cavity as assessed by hysterosalpingogram was normal in recurrent miscarriage 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 oestradiol concentrations, prolactin and progesterone concentrations greater than 10 ng/ml in mid-luteal phase), anatomical tests (hysterosalpingography and pelvic ultrasonography) and semen analysis (WHO criteria; WHO, 1999). 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 three portions. One piece was placed in 4% paraformaldhyde tissue fixative for 24 h 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. (1975). Human placental tissue was used as a positive control (Bhuiyan et al., 2006).

Quantitative immunohistochemistry

Endometrial specimens were cut into 6 μm sections using a Cryocut and placed on poly-L-lysin coated slides and stored at −70°C for immunostaining. A monoclonal mouse antihuman antibody (6H2, cat. no. 91205; Abcam, UK) was used to detect the intensity and distribution of eNOS immunostaining using standard immunohistochemical protocol. Briefly after rinsing the slides with buffer (0.1 mol/l phosphate-buffered saline (PBS), pH 7.4), endogenous peroxidases were quenched by incubation in 0.3% H_2O_2 in methanol for 15 min. Repeated rinses with 0.05% bovine serum albumin in PBS were performed followed by antigen retrieval using trypsin. Normal goat serum (1.5%; DAKO, Denmark) was then added to the slides in humidified chambers for 20 min 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 1 h. Slides were then rinsed three times with wash buffer, followed by incubation of sections with the secondary antibody, rabbit antimouse IgG, H and L (cat. no. ab6728; Abcam) diluted 1:1000 in PBS. Incubation with the secondary antibody was performed for 1 h at 37°C in an incubator. Slides were then exposed to 3,3-diaminobenzidine in H_2O_2 (DAKO) was for 15 min. Thereafter, the sections were counterstained with haematoxylin and mounted. In case of negative controls, a similar method was used but PBS 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). Six different areas of the sections were analysed at a magnification of ×40 and the mean was used for statistical analysis. The results were expressed as percentage integrated optical density.

Real-time reverse-transcription PCR

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. Total RNA (1 μg) was reverse transcribed into single-stranded complementary DNA (cDNA) with use of the Omniscript Reverse Transcription kit (GeneON, Germany) at 37°C for 60 min in a total volume of 20 μl. The reaction mix consisted of 1 ml of 10-fold diluted cDNA, qPCR MasterMix Plus for SYBR green I reagent (GeneON) and optimized forward and reverse gene-specific primers (300 nmol/l each). Reactions were run in triplicate in 96-well plates with a Mx3000P real-time PCR system (Stratagene, Santa Clara, CA, USA). The thermal cycling program was 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The internal control used was human hypoxanthine-guanine phosphoribosyltransferase (de Kok et al., 2005). Data were analysed 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 the control was subtracted from the CT value of eNOS gene to obtain a ΔCT value. The relative fold change for each gene was calculated with use of the ΔΔCT method (Livak and Schmittgen, 2001).

Statistical analysis

Results were analysed by ANOVA, comparing percentage integrated optical density for the immunohistochemical data and fold change in case of real-time reverse-transcription PCR using Statistical Package for Social Sciences software (SPSS, USA). Post-hoc analysis was performed using the Student–Newman–Keuls test. $P < 0.05$ was considered statistically significant.

Results

The demographics of the three groups of women studied are shown in **Table 1**. Women in the unexplained infertility group were significantly younger than the controls ($P = 0.036$). There were no differences in body mass index among the three groups. Four subjects in the control group and two in the recurrent miscarriage and unexplained infertility 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 (**Figure 1**). eNOS protein staining in glandular epithelium (**Figure 1A–C**) was less intense than in luminal epithelium (**Figure 1D–F**). Weak staining of eNOS was detected in the vascular endothelium (**Figure 1G**) and the stromal layer (**Figure 1H**). eNOS expression was higher in glandular epithelium of patients with recurrent miscarriage and unexplained infertility compared with control (**Figure 1A–C**). A similar pattern was found in the luminal epithelium only in patients with Recurrent Miscarriage (**Figure 1D–F**).

A summary of image analysis for eNOS expression in different layers of the endometrium is given in **Figure 2**. In women with recurrent miscarriage, glandular ($P = 0.004$) and luminal epithelial ($P = 0.002$) expression of eNOS was greater compared with controls, whereas stromal and vascular endothelial expression of eNOS was not significantly different for recurrent miscarriage and unexplained infertility as compared with the control group. In women with unexplained infertility, the expression of eNOS was only higher in luminal epithelium compared with controls ($P < 0.021$), with no significant differences in the other layers.

The expression of eNOS mRNA in the endometrium of women with recurrent miscarriage and unexplained infertility was significantly higher compared with controls ($P < 0.05$; **Figure 3**), confirming the protein data.

Discussion

The data confirm prior reports on endometrial eNOS immunolocalization (Khorram et al., 1999) and demonstrate a differential site-specific alteration in expression of eNOS protein in women with unexplained infertility and recurrent miscarriage 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 recurrent miscarriage and unexplained infertility groups in this area. The expression of eNOS mRNA in the endometrium of women with unexplained infertility and recurrent miscarriage was significantly higher compared with controls, thus confirming the protein data. The luminal eNOS protein changes in both recurrent miscarriage and unexplained infertility 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. (1996) demonstrated that the expression of NOS and prostaglandins E and F increase 1 day before implantation and that suppression of NOS by nitro-L-arginine methyl ester (L-NAME) decreased production of prostaglandins E and F_2 on the day of implantation in rats. Biswas et al. (1998) injected L-NAME into the rat uterine horn and demonstrated

Table 1 Demographic characteristics of the study population.

	Control (n = *10*)	*Recurrent miscarriage* (n = *10*)	*Unexplained infertility* (n = *10*)
Age (years)	36.1 ± 1.1	32.8 ± 1.9	29.8 ± 0.8[a]
Body mass index (kg/m^2)	26.1 ± 1.6	23.9 ± 1	25 ± 1
Live births	1.4 ± 0.2	0	0
Miscarriages	0	4.7 ± 0.7	0

Values are mean ± SEM.
[a]$P = 0.036$.

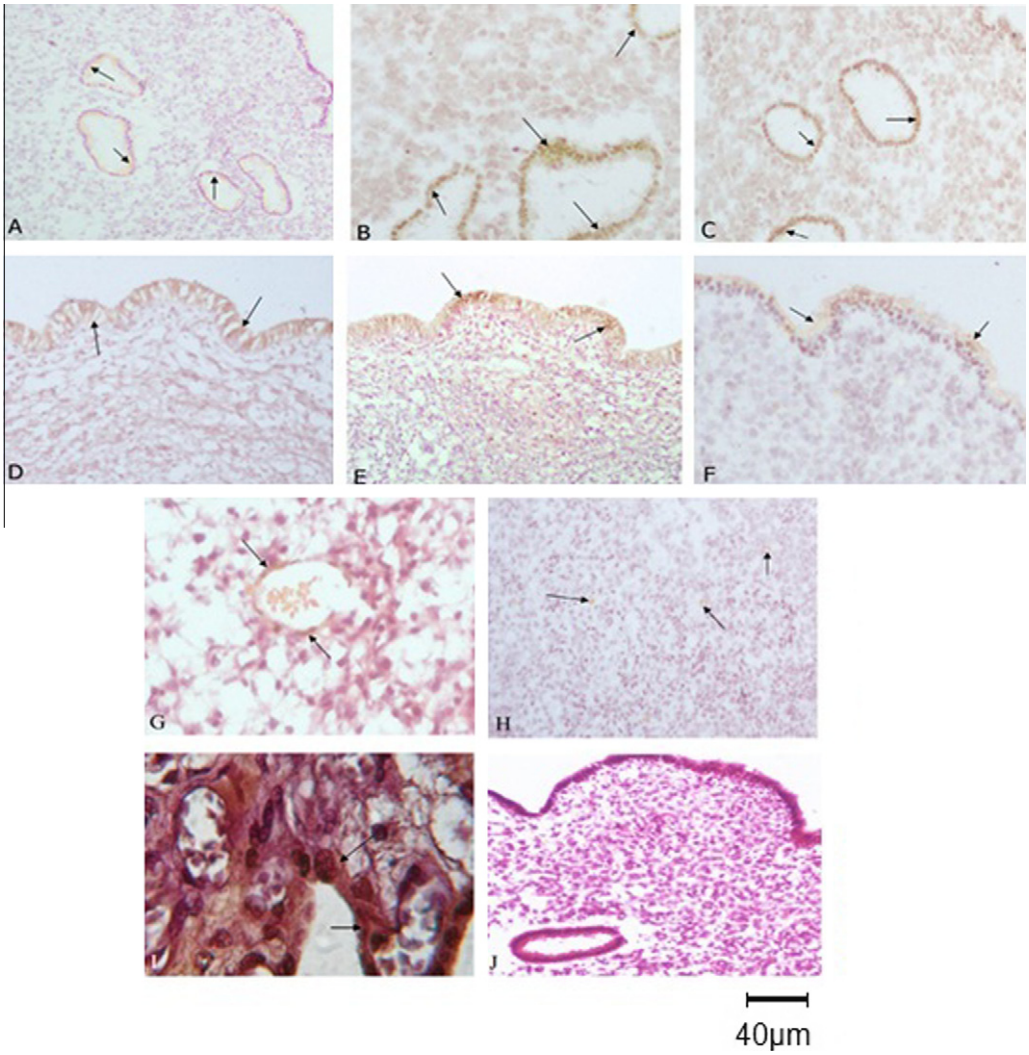

Figure 1 eNOS immunostaining in different endometrial compartments of the study population. Glandular epithelium (**A**—**C**) and luminal epithelium (**D**—**F**) of a control subject (**A**, **D**), recurrent miscarriage patient (**B**, **E**) and unexplained infertility patient (**C**, **F**) and vascular endothelium (**G**) and stroma (**H**) of a control subject. Negative (**I**) and positive (**J**) controls in a human placental section. Arrows indicate areas with positive eNOS immunostaining. The magnification used was ×40; bar = 40 μm.

an inhibition of implantation, and similar results were obtained by Duran-Reyes et al. (1999) using a different NOS inhibitor. In contrast to these studies, knockout mice for various isoforms of NOS do not show reduced litter size (Huang et al., 1993, 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 unexplained infertility (Koot et al., 2011) and recurrent miscarriage (Li et al., 2002b), the present study 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 recurrent miscarriage and unexplained infertility 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, so therefore localized excess NO production at the luminal surface could induce epithelial apoptosis and implantation failure. Vatansever et al. (2005) also reported increased eNOS immunoreactivity in the endometrium of unexplained infertility patients, although this was associated with a lower number of apoptotic cells. A second mechanism by which NO might impair implantation is through localized nitrosative stress. NO by virtue of its unpaired electron is a highly reactive free radical that, in excess, can damage protein, carbohydrates, nucleotides and lipids (Agarwal et al., 2008). Based on the present data, it is proposed that excess eNOS expression in luminal epithelium of patients with recurrent miscarriage and unexplained infertility can create local oxidative stress which could impair implantation, similar to other inflammatory gynaecological conditions such as endometriosis (Dong et al., 2001; Khorram and Lessey, 2002; Ota et al., 1998; Wu et al.,

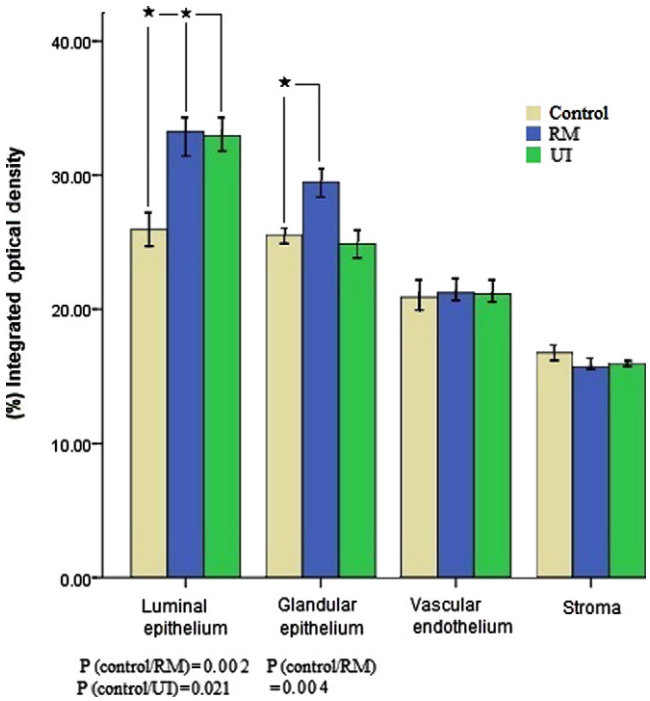

Figure 2 eNOS protein quantification by image analysis in four compartments of the endometrium in control, recurrent miscarriage (RM) and unexplained infertility (UI) groups. $^{*}P < 0.05$.

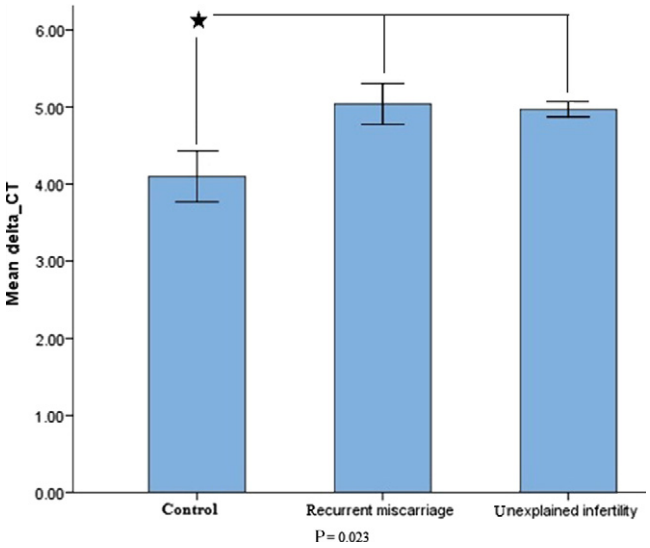

Figure 3 eNOS mRNA levels in whole endometrial tissue homogenates in control, recurrent miscarriage and unexplained infertility groups. $^{*}P < 0.05$.

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 antioxidants 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 recurrent miscarriage, and in so doing have examined polymorphism of the eNOS gene in different ethnic groups A recent meta-analysis of these studies showed a significant association in eNOS Glu298Asp polymorphism and recurrent miscarriage (Su et al., 2011). These genetic abnormalities could lead to reduced NO production and impaired endometrial function. The present data did not show any differences in endothelial microvascular eNOS concentrations in recurrent miscarriage and unexplained infertility 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 also that 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 these endometrial samples, could be a factor which can lead to infertility and miscarriage, these expression levels were not significantly different compared with 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, the present data demonstrate an overexpression of eNOS protein and mRNA in the endometrium of recurrent miscarriage and unexplained infertility. This aberrant pattern of eNOS protein and mRNA expression is similar to other inflammatory gynaecological 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 recurrent miscarriage and unexplained infertility is deleterious and could induce nitrosative stress. which could lead to implantation failures or failure of early pregnancy maintenance. More information about this can be obtained in the future, studying NO expression during full-term pregnancies, and it would also be helpful to compare the expression of eNOS in full and second-trimester pregnancies.

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