



# Immunohistochemical Localization of Endothelial Nitric Oxide Synthase in Endometrial Tissues of Women With Uterine Myomas

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## Abstract

**Objectives:** Nitric oxide (NO) is a free radical that plays important roles in variety of physiological aspects of the female reproductive system. Pathophysiological findings revealed a potential role of the endothelial isoform of nitric oxide synthase (eNOS) enzyme in female reproductive disorders specifically in the endometrium. This study investigates the expression of eNOS in the endometrial tissue to study the potential role of this enzyme and its NO production in infertility of women with uterine myomas.

**Materials and Methods:** A total of 20 endometrial tissues were obtained, 10 infertile women with uterine fibroids and 10 from normal and fertile women, 7 to 9 days after LH surge. Following fixation with paraformaldehyde, frozen sections of samples were prepared for semi-quantitative immunohistochemical evaluation using monoclonal anti-human eNOS antibody. Hematoxylin and eosin staining was used for histological dating of the samples

**Results:** Localization of eNOS was seen in glandular and luminal epithelium, vascular endothelium and stroma in both fertile women and infertile women with uterine fibroids. Despite the differences in immunoreactivity of luminal epithelium, vascular endothelium and stroma in both groups, higher levels of eNOS in glandular epithelium was statistically significant in women with uterine fibroids compared to the control group.

**Conclusions:** The findings suggest that over-expression of eNOS in glandular epithelium may affect the preparation stage of endometrium for fertility in women with uterine myomas.

**Keywords:** Nitric oxide, Endothelial nitric oxide synthase, Endometrium, Uterine myomas, Fibroids

## Introduction

Uterine myomas or fibroids are known as the most prevalent benign solid tumors of the female reproductive system. They are commonly found in the myometrial layer of the uterus; however their presence in other different locations both inside and outside the uterus has been reported. Although myomas are very prevalent and are diagnosed in 25%–50% of females, the exact pathogenesis has not been determined; however, it has been reported that these tumors are derived from myoma cells by the intervention of specific hormones (1). Uterine myomas can diversely affect normal pathophysiology of reproductive system and cause pain, infertility, pregnancy loss, menorrhagia and many other complications through different mechanisms. Moreover, they could reduce the efficacy of assisted reproductive technologies and the implantation rate (2). Studies have proven that intramural and submucosal fibroids could lower the rate of fertility (3). Since 1980, surgical myomectomy is routinely used as a common treatment procedure for patients with uterine myomas; this technique has been approved for its outstanding advantages in medical, social and economic

terms by decreasing morbidity (4). However, in many cases, due to their asymptomatic nature, uterine myomas do not necessarily need to be removed.

Nitric oxide (NO) is a free radical that plays diverse physiological roles in human systems. Nitric oxide synthase (NOS) is the name given to a group of enzymes which produce NO from L-arginine amino acid. In the human endometrial tissue, all three isoforms of NOS (iNOS, eNOS and nNOS) are expressed; However the endothelial isoform (eNOS) is the predominant one(5). To date, we do know eNOS acts as a regulator of uterine quiescence during the gestational period and studies have demonstrated its role in signal transduction pathways (6) as well as relaxation of myometrial smooth muscles (7,8). Even though the release of eNOS is a critical factor to maintain pregnancy, over-expression of this enzyme has been shown to induce cellular apoptosis (9) and/or impair endometrial and myometrial function by nitrosylation of key endometrial proteins (10,11) as well as subsequent effects on myometrial relaxation. The same conditions also have been reported in the state of endometriosis (12-14), adenomyosis (15), unexplained infertility, and

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recurrent miscarriage (16). It has been demonstrated that estrogen stimulates the proliferation of the uterine fibroids by direct activation of the uterine fibroblast cells (17). Additionally, studies have shown that estrogen and progesterone are the major regulators of expression of eNOS in the human endometrium (18-20). Thus, in regard to estrogen-dependent pathology of the uterine fibroids, it seems that NO levels may play a considerable role in the formation of uterine fibroids. These alterations in the enzyme expression, can lead to formation of oxidative stress, which has been shown to cause reproductive disorders (21,22). Accordingly, we hypothesize that over-expression of endometrial eNOS could occur in patients with uterine myomas and potentially affect their fertility. The role of NO in uterine myomas has been studied by a few studies; however all of those have focused on the fibroid tissues per se as well as myometrium and thus the potential resultant endometrial alteration of the enzyme remained unclear (23,24)

As the main expressed isoform of NOS in musculature and endothelium of myometrial vessels, eNOS can be potentially involved in the pathophysiology of endometrial layer. Endothelial nitric oxide synthase (eNOS) plays an imperative role in the maintenance of uterine quiescence and NO produced by the action of this enzyme can be involved in diverse physiological aspects of gestation. Therefore, we sought to clarify whether expression of eNOS is altered in patients with uterine fibroids and whether (if any) it can influence the process of implantation.

## Materials and Methods

### Study Design

The study group was asked to sign a consent form for their participation. In this randomized study, women in the control group (n=10) with no history of urogenital conditions including uterine myomas and endometriosis experienced at least one natural labor and had normal menstrual cycles and had not received any hormonal medications for at least 4 months before sample collection. This women voluntarily participated in this study upon their attendance for regular process of gynecological health check and mean age of those women was 33.9 years (range, 29–39 years). Uterine fibroids in myometrium of experiment group (n=10) had been diagnosed by ultrasonography and hysterosalpingography (6 intramural, 4 submucosal). Within this group, patients who had reasons of infertility other than uterine myomas such as endometriosis, adenomyosis, abnormal endocrine findings (TSH, cycle-day-3 FSH and estradiol concentrations, prolactin and progesterone concentrations) were excluded from the study. The study group was selected from among those women with uterine myomas, which resumed their fertility after myomectomy by report of at least one successful pregnancy and regardless of miscarriage to ensure that their infertility was truly myoma-based or at

least in very close relationship with fibroid formation.

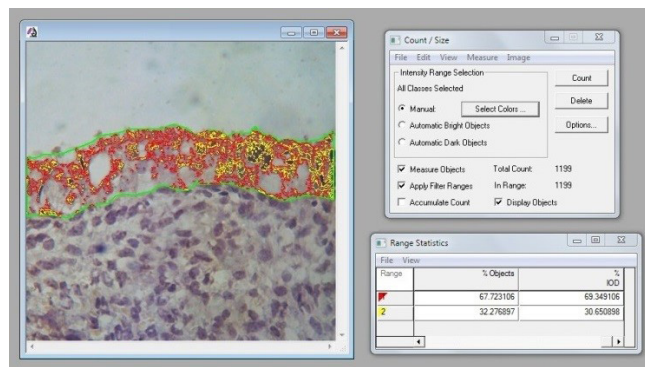
### Sample Collection and Preparation of Sections

Biopsy samples of endometrium were obtained by using a Pipelle curette 7 to 9 days after the urinary LH surge at the sites close to the myomectomy following Cuntz procedure (25). Since the plan of this study was to analyze the eNOS change in patients with uterine fibroids from the implantation point of view, all of the phases of the post-ovulatory samples were determined by ultrasound to ensure about the correct timing of sample collection. At first obtained samples were treated with 4% paraformaldehyde tissue fixative for maximum 24 hours followed by rinsing in 70% ethanol and later stored at -20°C until processing. Histological dating was done by using the criteria of Noyes et al (26). Each biopsy specimen was later cut into 6 µm sections by using a Reichert-Jung Cryocut 1800 (Cambridge Instruments GmbH, Nussloch, Germany) and place on poly l-lysine coated slides and store in -70°C until immunostaining.

### Quantitative Immunohistochemistry

For microscopic detection of the intensity and distribution of eNOS immunostaining, a monoclonal mouse antihuman antibody (6H2, cat. no. 91205; Abcam Co, USA) was applied. Rinsing with buffer (0.1 M of phosphate-buffered saline with a pH of 7.4) followed by incubation in 0.3% H<sub>2</sub>O<sub>2</sub> in methanol for 20 minutes to quench endogenous peroxidases. Rinsing repeated with 0.05% bovine serum albumin in phosphate-buffered saline and retrieval of antigens was done by adding trypsin. Afterwards, the slides were exposed to 1.5% normal horse serum (Sigma Chemical Co., St. Louis, MO) in humidified chambers for 30 minutes at room temperature. The primary antibody against eNOS (1:100) was placed on slides and incubated at 37°C for 1h. After binding of the primary antibody, the sections were incubated with the secondary antibody, a rabbit antimouse IgG (Abcam Co. USA) diluted in PBS. Incubation with the secondary antibody was performed for 1h at room temperature. 3,3'-diaminobenzidine in H<sub>2</sub>O<sub>2</sub> (Vector Laboratories, Inc.) was added to the sections for 10 minutes. Thereafter, the sections were counterstained with hematoxylin and mounted. For negative controls, using same method, phosphate-buffered saline replaced the primary antibody.

Immunohistochemical staining was evaluated quantitatively by 2 independent examiners by using Image-Pro Plus software (Figure 1) using previously described methods (27) on figures of Zeiss light microscope (Axioskop, Zeiss, Göttingen, Germany). Four different areas of the sections were analyzed at varying magnifications and the mean was used for statistical analysis. To avoid errors due to uneven staining, 2 sections of each endometrial biopsy specimen including both epithelial and stromal cells were evaluated. We used human placental sections as positive controls which



**Figure 1.** Image Pro Plus software used to determine staining intensity of sections. immunohistochemical staining reported as intensity of optic density (IOD%).

showed normal immunostaining (28).

### Statistical Analysis

Differences in immunohistochemical staining between control and experimental groups as intensity of optic density (IOD%) were analyzed by using the exact Man-Whitney U test of SPSS software. Results have been reported as mean standard deviation and  $P \leq 0.05$  was considered statistically significant.

### Results

#### eNOS Immunolocalization in Endometrium

The demographics of the 2 groups of study are shown in Table 1. None of the subjects had any chronic medical illness and laparoscopic evaluation and hormonal tests showed normal anatomy and endocrinology of all subjects. There was also a small difference in age and body mass index between two groups which were not statistically significant. eNOS was localized in vascular endothelium, glandular epithelium, luminal epithelium and the stroma (Figure 2). Expression of eNOS in endometrium of infertile women changed in vascular endothelium ( $P=0.65$ ), glandular epithelium ( $P=0.045$ ), luminal epithelium ( $P=0.44$ ), and stroma ( $P=0.60$ ).

Accordingly, despite the differences between expression of eNOS in luminal epithelium, stroma and vascular endothelium only the difference between two groups in glandular epithelium was statistically considerable ( $P=0.045$ ; Figure 3).

### Discussion

Known as the most common benign uterine tumor in uterus of women of reproductive age, uterine myomas

or fibroids are hormone dependent (especially estrogen and progesterone) tumors that manifest largely in the myometrial layer of uterus and in most cases have to be extruded surgically due to their approved negative effects on reproductive health based upon the location and physical characteristics (29).

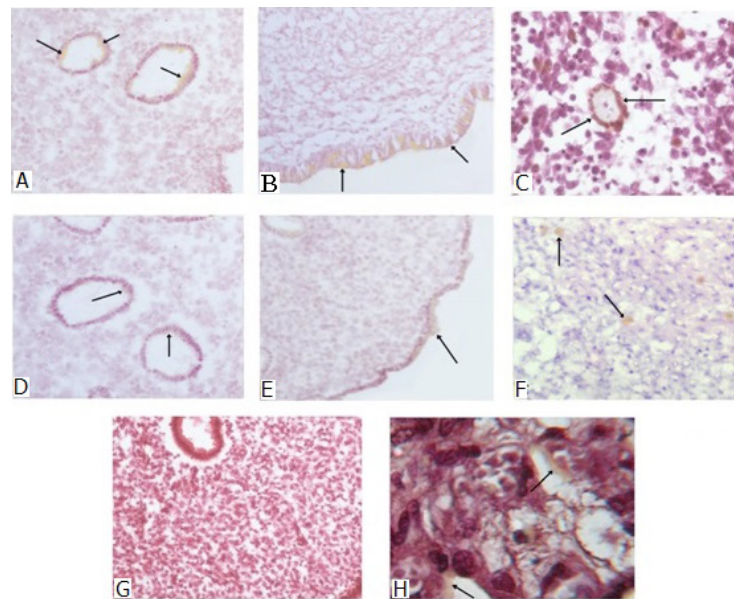
We detected eNOS in blood vessels as well as glandular and luminal epithelial cells and endometrial stroma. These findings are in accordance with previous studies by Najafi et al (16) and Tefer et al (30). It has also been reported previously that at the time of expected implantation, the endothelial isoform of NOS is expressed dominantly in the human endometrium (18). By continues release of NO into the lumen, processes such as menstruation and implantation are facilitated through prostaglandin synthesis and modulation of anchoring proteins. On the other side, eNOS-derived NO, may function as an inhibitor of endometrial platelet aggregation by activating the soluble guanylyl cyclase formation or through cyclooxygenase catalysis (31).

Our previous studies showed that glandular epithelium releases most eNOS from the endometrial tissue in normal and infertile women with unknown etiologies (16,32). This finding was confirmed in the present work. Although this finding is partially in contrast with that of Taguchi (33), reporting that vascular endothelium is the major part of eNOS expression, we see a small increase in the endothelial expression of the enzyme in the vessels. This increased local NO expression could cause activation of cyclooxygenase-2 and subsequently elevating the prostaglandins levels, such as prostaglandin E2 (34). On the other hand, the vascular expression of eNOS in our study, approves the findings by Gokdeniz et al,

**Table 1.** Characteristics of study group (n=10) and control group (n=10)

	Age (y)	BMI (kg/m <sup>2</sup> )	Live Birth (n) *	Infertility (y) *	Cigarette Smoke	No. of Pregnancy Prior to Myomectomy	Number of Pregnancy Following Myomectomy
Control	33.9 SD=1.8	24.59 SD=2.3	1.60 SD=2.1	0	0	2.8 SD=1.8	N/A
Uterine myomas	32.3 SD=1.6	24.05 SD=2.00	0.00	3.10 SD=2.0	0	0	1.3 SD=2.00

Abbreviation: SE = standard error.



**Figure 2.** Immunostaining of eNOS in different endometrial compartments of the study groups. Glandular epithelium (A) and luminal epithelium (B) and vascular endothelium (C) of a myoma. Glandular epithelium (D), luminal epithelium (E) and stroma (F) of a control subject. Negative (G) and positive (H) controls in a human endometrium and placental sections respectively. Arrows indicate areas with positive eNOS immunostaining. Magnification for epithelium is 10 (A, B, D, E, G) and for endothelium and stroma is 40 (C, F, H)

nevertheless their study mainly focuses on the muscular characteristics of myoma tissues (35)

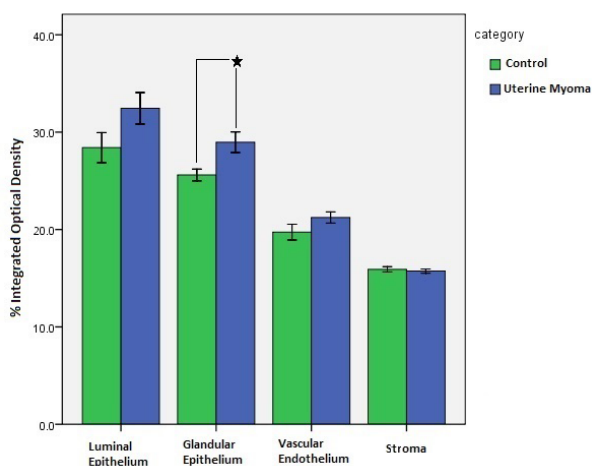
Although this study shows a non-significant increase in the expression of enzyme in vascular endothelium, this finding is not in contrast with results of Oh et al study (36) which found significant over-expression of enzyme in vasculature of the endothelium. Vascular over-expression of eNOS in case of uterine myoma may affect the biological integrity of endometrium. It has been shown that unpaired electron of extremely reactive NO molecule

in higher amounts, are potentially able to damage the structure of protein, carbohydrates, nucleotides and lipids (21). Accordingly for uterine fibroids, we can assume a mechanism similar to that which occurs in inflammatory conditions such as endometrial adhesions by inducing oxidative stress. But due to different reported amount of the enzyme, it seems that the expression of eNOS in endometrial vessels varies by location and/or menstrual date.

Apart from the vessels wall, the presence of eNOS in luminal epithelium and stromal cells of women with uterine fibroids may be the indicative of the role of NO in the control of endometrial function. But based on the findings of this study we cannot claim that if pregnancy occurs, even for a short duration of time, this role may be interrupted. It seems that like endometrial tissues of women with unexplained infertility and recurrent pregnancy loss, the cyclo-oxygenase pathway, NO and cytokines which interact to regulate uterine function (34) have also been undisrupted in women with uterine fibroids.

Although NO in its physiological release, acts specifically on the myometrial tissue to cause muscle relaxation at the end stage of labor, changes in the endometrial expression of eNOS, as the major isoform of NOS in the females' reproductive tract, can demonstrate the activity log of this free radical in the lumen of uterus in specific malignancy conditions.

From the window of implantation, it has been strongly reported that uterine fibroids could affect various stages of fertility especially early implantation which can result in infertility and miscarriage (3). Several studies



**Figure 3.** eNOS Protein Quantification by Image Analysis In 4 Compartments of the Endometrium in Control, and Uterine Myoma Groups. The expression of eNOS in glandular epithelium has been significantly increased. On the other hand, in spite of relatively large differences in luminal epithelium, the higher standard errors in this group indicate the insignificance of the difference by Mann Whitney U test. \* $P < 0.05$ . Error Bars=  $\pm 1$  SE.



have demonstrated that increase in the expression of prostaglandins E and F and NO occurs as complementary supplements of implantation (37,38) and from this point of view, we can suggest that due to infertility outcomes of uterine fibroids, the increase in glandular epithelium may act as a compensatory mechanism to overcome unwanted results of uterine myomas on implantation and embryo-endometrial adhesions. But further studies are needed to investigate if suppression of eNOS activity by nitro-L-arginine methylester (L-NAME), a common NOS blocker, can cause the changes in the implantation and fertility of patient with uterine myomas. Taking the implantation failure secondary to endometrial factors in unexplained infertility (39) and recurrent miscarriage (40) into consideration, the present study suggests that an irregular endometrial expression of eNOS in women with uterine fibroids may result in implantation failure through the similar mechanisms.

The expression levels of eNOS in vascular endothelium were not significantly different compared to the control group, however, the low expression of the enzyme, as seen in these endometrial samples, could be considered as a potential factor leading to infertility and miscarriage subsequent to myoma formation. This suggests that aberrant production of eNOS in non-endothelial areas (especially glandular epithelium) may play a more remarkable role in the pathophysiology of uterine fibroids compared to endothelial areas. Although we used specialized software to detect fold changes in enzyme expression, it can be more useful to confirm this data with quantitative protein analysis such as blotting techniques, which may be considered as a limitation of our study due to difficulties in collection of endometrial samples.

In summary, the present data by demonstrating an over-expression of eNOS in the endometrium of fibroid uteri, suggests that excess expression of eNOS (and subsequent excess generation of NO in the endometrium) in patients with uterine myomas may cause nitrosative stress which in turn could result in infertility or miscarriage or even preterm birth; However we do know that expression of eNOS to some extent is essential for implantation and fertilization. It seems that this disordered expression of eNOS is similar in inflammation pattern to other gynecological conditions such as endometriosis and adenomyosis and can be considered as a potential component resulting in infertility and/or miscarriage. However, larger and comprehensive studies with concentration on NOS protein and gene expressions are needed to explore exact mechanisms of NO action in the endometrial tissue in these women.

#### Conflict of Interests

Authors declare that they have no conflict of interests.

#### Ethical Issues

The protocol for this randomized study was approved

by the Ethics and Human Subjects Committee at Shahid Beheshti University of Medical Sciences (91st Session, 2011).

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