—Mini Review—

Mechanism Underlying the Low Implantation Rate in Patients with Thin Endometrium

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Abstract: *The aim of this study was to clarify the mechanism underlying the low implantation rate in patients with thin endometrium. Endometrial thickness during postovulation days 5–7 (mid-luteal phase) during a total of 1,035 natural cycles in 205 patients was analyzed retrospectively. We designated patients with endometrial thicknesses ≤ 6 mm as the thin group (n = 12) and those with endometrial thicknesses ≥ 7 mm as the normal group (n = 193), based on the markedly lower pregnancy rate in the thin group (thin: 8.3% vs. normal: 51.3%). Levels of steroid receptor, transforming growth factor α (TGFα) and oxidative stress were compared between the two groups. Oxidative stress was higher in the stroma of thin endometrium. In addition, expression of progesterone and estrogen receptors was higher, and TGFα expression was significantly lower in thin endometrium. Altered regulation of oxidative stress and levels of steroid receptors and TGFα appear to underlie the low implantation rate seen in patients with thin endometrium.*

Key words: *Thin endometrium, Oxidative stress, Steroid receptors, TGFα*

Introduction

Since 1989, when it was first reported that the endometrium is significantly thicker in pregnant women than in the nonpregnant women [1], there have been numerous studies on the impact of endometrial thickness on the pregnancy rate. Most of these studies have shown that abnormally thin endometrium is associated with low rates of pregnancy [2–4]. However, the pathophysiology of the low implantation rate in patients with thin endometrium is not well understood. In the present study, we analyzed clinical data, levels of oxidative stress, and levels of steroid receptor and transforming growth factor α (TGFα) expression to further clarify the mechanism underlying the low implantation rate among patients with thin endometrium.

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Materials and Methods

Patients

A total of 205 infertile women accounting for 1,035 natural menstrual cycles (mean age, 35.3 ± 4.2 years; range, 22–45 years) were recruited into the study. Endometrial thickness was estimated by transvaginal ultrasonography using a NEMIO SSA-550A system with a PVM-621VT (6.0 Mhz) vaginal transducer (Toshiba, Tokyo, Japan), and was defined as the maximum distance between the echogenic interfaces of the myometrium and endometrium measured in a plane through the central longitudinal axis of the uterus. Endometrial thickness was expressed as the average thickness on postovulation days 5–7 (mid-luteal phase) during 4–6 natural cycles of each patient. The day of ovulation was determined based on urinary LH assayed every morning and ultrasonography of the follicles. Blood samples were also taken for hormone analysis on the day of ultrasonography.

Endometrial biopsy

Informed consent was obtained from each patient before collection of tissue samples. Using a curette (J.A.M.W Uterine Curette, Tokyo, Japan), endometrial biopsy specimens were collected from the anterior wall of the uterine cavity without dilatation during the mid-luteal phase.

Immunohistochemistry

For immunohistochemical examination, tissue specimens were fixed in 10% formalin for a maximum of 24 hours, embedded in paraffin and cut into 4-μm-thick sections. Immunohistochemical studies were performed using an avidin-biotin peroxidase system with a Ventana-Bio Tel Techmate 500 automated immunostainer and a standard ChemMate ABC detection kit (Ventana Medical Instruments, Tokyo, Japan). Labeled sites were visualized using 3,3'diaminobenzidin as the chromogen.

Oxidative damage was assessed based on nuclear staining with monoclonal antibodies against 8-hydroxy-deoxyguanosine (8-OHdG) (Nihon Yushi, Japan) and 4-hydroxy-2-nonenal (4-HNE) (Nihon Yushi, Japan). Endometrial synthesis was graded on a scale of 1–4 based on the percentage of cells showing positive nuclear staining, as follows: 1, 0–25%; 2, 26–50%; 3, 51–75%; and 4, 76–100%.

Real-time PCR

Total RNA was extracted from tissue samples using an RNeasy Max kit (Qiagen, USA) and subjected to real time PCR using Superscript™ II RNaseH-Transcriptase (Invitrogen, Japan) with the following primers: for estrogen receptor 1, 5'-GCTGCCTCTATTATGGCACTTC-3' (forward) and 5'-GTTGTTTCTAGGGTGAGC-3' (reverse); for estrogen receptor 2, 5'-GGAGTTGGTACA-CATGATCAGC-3' (forward) and 5'-ACCTTGAAGTAGT-TGCCAGGAG-3' (reverse); for progesterone receptor, 5'-CTGTGGGAGCTGTAAGGTCTTC-3' (forward) and 5'-CCAGTGCTCTCACAACTCTGAC-3' (reverse); and for TGFα, 5'-AGGAGAATTTGTGCTTCTGGAG-3' (forward) and 5'-GCATTTGAGTCATTCCTCCTTC (reverse). Glyceraldehyde-phosphate dehydrogenase (GAPDH) served as an amplification control. Roche LightCycler FastStart DNA Master SYBR Green was used for fluorescence labeling, and the real-time PCR was carried out using a Roche Diagnostics Light Cycler Quick System.

Statistical analysis

Student's *t* test, Welch's *t* test and the Mann-Whitney U test were used for analysis of the data from patients with thin and normal endometrial thicknesses. Values of $P < 0.05$ were considered significant.

Results

The average endometrial thickness measured on postovulation days 5–7 during 4–6 natural cycles was ≥ 7 mm in 193 (94.1%) of the 205 patients studied. Following treatment that included assisted reproductive technology, 99 (51.3%) of those patients became pregnant. By contrast, only one (8.3%) pregnancy was achieved who's endometrial thickness was 6.2 mm among the 12 (5.9%) patients with endometrial thicknesses ≤ 6 mm ($P < 0.01$). We therefore designated patients with endometrial thicknesses ≤ 6 mm as the thin group and those with endometrial thicknesses ≥ 7 mm as the normal group.

The mean ages of the women in the thin and normal groups did not significantly differ (thin, 37.1 ± 4.7 vs. normal, 35.1 ± 4.2), nor did their serum estradiol and progesterone levels during the mid-luteal phase. When we assessed oxidative stress based on immunohistochemical analysis of nuclear staining of 8-OHdG and 4-HNE, we found that all cells in the glands and in the luminal epithelium were stained. By contrast, only a fraction of the stromal cells were stained, and the numbers of stromal cells positive for 8-OHdG and 4-HNE were significantly higher in thin endometrium (Fig. 1). Immunohistochemistry of estrogen and progesterone receptors and TGFα showed positive staining in nuclei of endometrial cells (Figs. 2, 3). Real-time PCR revealed that the expression levels of estrogen receptors 1 and 2 and progesterone receptor were all higher in the thin group than the normal group during the mid-luteal phase, while expression of TGFα was significantly lower in the thin group (Table 1).

Discussion

Although there have been reports that there is no correlation between endometrial thickness and pregnancy rates [5, 6], most studies have shown low pregnancy rates among patients with abnormally thin endometrium, and it is widely accepted that there is a minimum thickness necessary to establish a pregnancy; thicknesses ranging from 5 to 8 mm have been reported as thresholds for pregnancy [7, 8]. In the present study, we found that a minimum endometrial thickness of 6 mm was required to achieve pregnancy, which is in agreement with the threshold suggested by Gonen in 1990 [9]. However the mechanism underlying the low implantation rate in patients with thin endometrium is not well understood.

Our immunochemical assessment of oxidative damage to DNA and lipids using 8-OHdG and 4-HNE, respectively, revealed substantial positivity for both substances in the stroma of thin endometrium. This observation is clinically important because it suggests that in addition to making the thin endometrium thicker, the ultimate treatment for thin endometrium would also

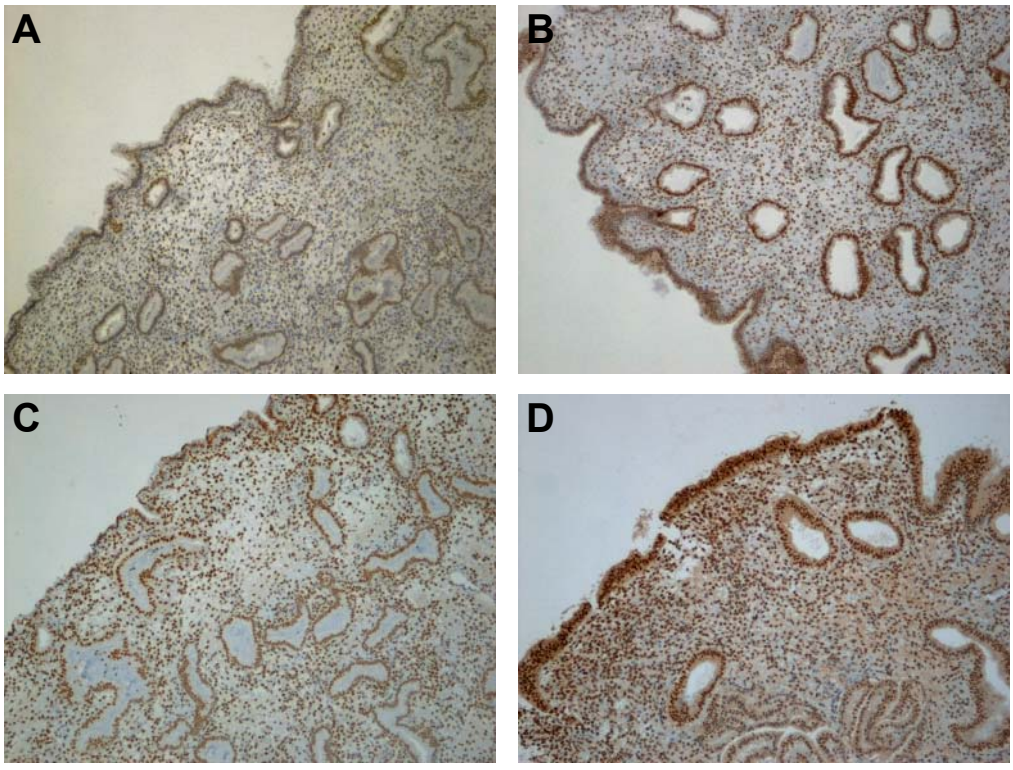

Fig. 1. 8-OHdG staining in normal endometrium (A) and thin endometrium (B). 4-OH nonenal staining in normal endometrium (C) and thin endometrium (D). Both luminal and glandular epithelium were stained positively for 8-OHdG and 4-OH nonenal, whereas only a fraction of the stromal cells were stained. Therefore, immunohistochemical scoring was chosen for analysis. The numbers of stromal cells positive for 8-OHdG and 4-OH nonenal were significantly higher in thin endometrium.

Table 1. Oxidative damage and levels of steroid receptors and TGFα in thin endometrium

Methods	Findings	Normal group	Thin group	*P* value
Ultrasound	Endometrial thickness	11.4 ± 1.7 (n = 193)	5.4 ± 0.7 (n = 12)	$P < 0.01$
Blood analysis	Estradiol	89.2 ± 62.2 pg/ml (n = 36)	92.8 ± 70.2 pg/ml (n = 9)	ns
	Progesterone	14.6 ± 5.4 ng/ml (n = 36)	13.8 ± 4.9 ng/ml (n = 9)	ns
Immunohisto-chemistry	8-OH deoxyguanosine	Score 2.4 (n = 10)	Score 3.4 (n = 7)	$P < 0.01$
	4-OH-2 nonenal	Score 2.1 (n = 10)	Score 3.1 (n = 7)	$P < 0.01$
RT-PCR	Estrogen receptor 1	0.4 ± 0.8 F (n = 13)	0.59 ± 0.9 F (n = 7)	ns
	Estrogen receptor 2	1.12 ± 1.4 F (n = 13)	6.54 ± 8.3 F (n = 7)	ns
	Progesterone receptor	0 F (n = 11)	0.54 ± 0.7 F (n = 7)	ns ($P = 0.07$)
	Transforming growth factor α	14.1 ± 6.9 F (n = 11)	4.8 ± 7.9 F (n = 7)	$P < 0.01$

Endometrial thickness was observed using ultrasonography. Analysis of hormone levels, immunohistochemistry and PCR were performed on post-ovulation days 5–9 (mid-luteal phase). Oxidative damage was assessed based on generation of 8-OH deoxyguanosine and 4-OH-2 nonenal. Immunohistochemical scores of 1–4 were assigned based on the percentage of cells showing positive nuclear staining, as follows: 1, 0–25%; 2, 26–50%; 3, 51–75%; and 4, 76–100%. Levels of ER1and 2, PR and TGFα were determined based on fluorescence scores following PCR.

need to suppress oxidative damage. Consistent with that idea, Ledee-Bataille *et al.* [10] showed that pentoxifylline and tocopherol, two vasodilators that also exert antioxidant effects, were effective for treating patients with thin endometrium. Consistent with their report, eight of the 15 patients whose endometrial

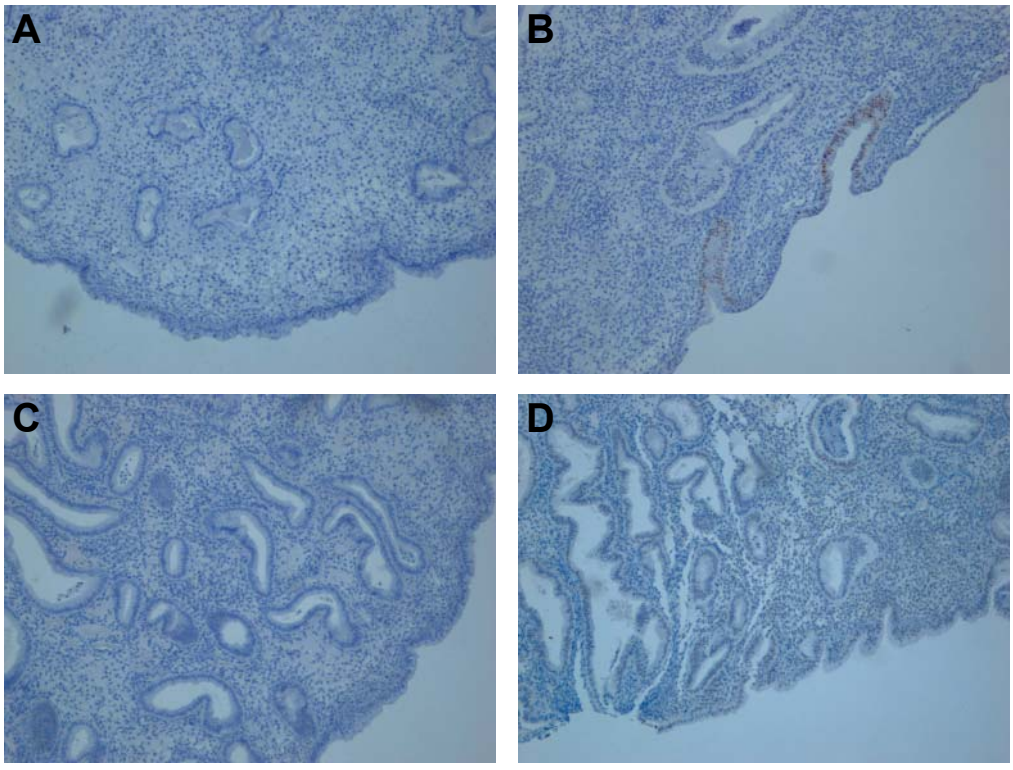

Fig. 2. ER staining in normal endometrium (A) and thin endometrium (B). PR staining in normal endometrium (C) and thin endometrium (D). These steroid receptors are normally downregulated during the mid-luteal phase, but downregulation tended to be incomplete in thin endometrium.

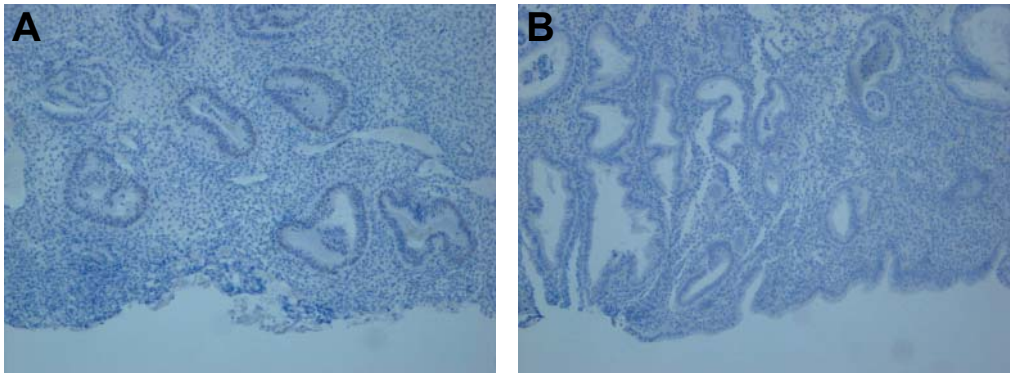

Fig. 3. TGFα staining in normal endometrium (A) and thin endometrium (B). Immunohistochemical staining for TGFα was generally weak and no specimen of thin endometrium was stained.

thickness was < 6 mm during the implantation period became pregnant after treatment with pentoxifylline, tocopherol and ascorbic acid at Tonan Hospital. In contrast, no pregnancies were achieved without the treatment.

It is known that estrogen receptors 1 and 2 and progesterone receptors are downregulated during the mid-luteal phase [11]. All three receptor types are induced by estrogen, and their disappearance during the luteal phase is believed to reflect the cumulative activity of progesterone [12]. We found that although the difference was not significant, there tended to be

less downregulation of estrogen and progesterone receptors in the thin group. Apparently, however, these changes in the thin endometrium do not reflect differences in serum steroid hormone levels, which did not differ between the two groups.

TGFα reportedly supports the growth of endometrial stem cells, which are thought to be important mediators of endometrial regeneration [13]. It is therefore noteworthy that we found expression of TGFα to be significantly downregulated in thin endometrium.

We previously reported that traumatic damage to the endometrium caused by dilatation and curettage (D&C) likely plays a key role in the etiology of thin endometrium [14]. Given our present findings, we would suggest that increased oxidative stress and altered regulation of steroid receptor and TGFα expression are the result of functional damage to the endometrium caused by D&C, thereby contributing to the low implantation rate in patients with thin endometrium.

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