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Endometrial Glycodelin-A Expression in Patients with IVF Failure

IVF Başarısızlığı Olan Hastalardaki Endometriyal Glikodelin Ekspresyonu

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ABSTRACT

Objective: We aimed to assess the expression of glycodelin-A in the midluteal secretory en-dometrium of women with previous in-vitro fertilization (IVF) failure. **Material and Methods:** Our study is a retrospective case controlled study. Patients who had at least one IVF failure despite transfer of at least 2 good quality embryos (Group 1) and conceived in their subse-quent IVF attempt (Group 2 (n: 13)) were included. Endometrial biopsies were taken be-tween days 17-19 of their natural cycle prior to IVF treatment. Immunohistochemical staining with Gd-A antibody was performed and H-Score was provided accordingly. **Results:** There was statistically significant difference between the groups regarding Gd-A expression in lu-minal epithelium (114.0±32.7 vs. 210.9±84.6, P=0.004) and glandular epithelium (141.5±68.2 vs. 218.3±105.9, P=0.040). When the indication for IVF was male factor, Gd-A staining was similar between the groups; however, when the indication was female factor or unexplained infertility, the H-SCORE for Gd-A staining was statistically lower in Group I compared to Group II. H-SCOREs showed statistical significance in luminal and glandular epithelium H-SCORE, but best predictor was found to be luminal epithelium H-SCORE. **Conclusion:** Midluteal endometrial Gd-A expression can be used for the prediction of IVF success.

Key Words: Glycodelin, endometrial biopsy, IVF failure

ÖZET

Amaç: İn vitro fertilizasyon (İVF) başarısızlığı olan kadınların midluteal sekretuar endometriumunda glikodelin-A (Gd-A) ekspresyonunu belirlemek. **Gereç ve Yöntemler:** Çalışmamız, retrospektif vaka kontrollü çalışmadır. Çalışmaya, 2 adet iyi kalite embryo verilmesine rağmen İVF başarısızlığı olan (Grup 1 (n:12)) ve İVF sonrası gebelik elde edilen (Grup 2 (n:13)) hastalar dahil edildi. Endometriyal biyopsi, İVF öncesi doğal siklusun 17-19. günlerinde alınarak, Gd-A antikoru ile immünhistokimyal boyama yapıldı ve H-skorları elde edildi. **Bulgular:** Gruplar arasında, endometriyal luminal epitel (114.0±32.7 vs. 210.9±84.6, P=0.004) ve glandüler epitel (141.5±68.2 vs. 218.3±105.9, P=0.040) midluteal Gd-A açısından istatistiksel olarak anlamlı fark bulunmuştur. Erkek faktörü nedeniyle İVF uygulanan hastalarda midluteal Gd-A ekspresyonu açısından istatistiksel fark bulunmamıştır fakat kadın faktörü ya da açıklanamayan infertilite nedeniyle İVF uygulanan hastalarda Gd-A boyanması ile ilgili H-skoru grup 1'de grup 2'ye göre daha düşük bulunmuştur. Luminal ve glandüler epitel H-skor'u istatistiksel olarak anlamlı olmasına rağmen, luminal epitel H-skor'u daha belilirleyici olarak bulunmuştur. **Sonuç:** İVF başarısını öngörmede midluteal endometriyal Gd-A ekspresyonu belirleyici bir faktör olarak kullanılabilir.

Anahtar Kelimeler: Glikodelin, endometriyal biyopsi, IVF başarısızlığı

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Despite the advances in in-vitro-fertilization technology, some patients repeatedly fail to conceive. There is yet no strict definition of recurrent IVF failure or recurrent implantation failure (RIF): a recent definition includes RIF as failure of implantation in at least three consecutive IVF attempts, in which one to two embryos of high-grade quality are transferred in each cycle. The causes of RIF may be related to reduced endometrial receptivity, undetected embryo defects or multifactorial causes.[1-3]

Evaluation of endometrial receptivity via morphological features or biomarkers may help many patients with RIF. Endometrial receptivity is crucial to embryo implantation. It involves a synchronization process with cascading events in the "implantation window" to prepare to harness the implanting embryo. Most investigations have focused their attention on the presumed window of implantation in the midluteal phase (cycle days 19-23).[4,5] In addition, multiple cytokines and proteins have been identified to be involved in the implantation process during the mid luteal phase including cellular adhesion molecules such as integrins, selectins and cathedrins, growth factors (Leukemia inhibiting factor), nuclear transcription factors (HOXA 10) cytokines, lipids and secretory proteins such as Glycodelin-A(Gd).[6]

Gd-A is secreted into endometrial cavity from decidual glands, but also found in fallopian tubes, ovary, mammary glands, vesicular glands and bone marrow.[7,8] Gd-A is increasingly secreted after day 18 of the menstrual cycle. Gd-A is supposed to have immunosuppressive effect on natural killer cells to allow implantation and to protect it from maternal immunity. If conception ensues, its serum levels increase rapidly and reach to a maximum at 8 to 10 weeks of gestation. Recently it has been shown to be significantly lower in women with miscarriage and in women with septate uteri.[9,10]

In this study, we aimed to assess the expression of Gd-A in the secretory endometrium of women with implantation failure.

MATERIALS AND METHODS

Patients who applied for IVF to Baskent University Ankara Hospital IVF unit were included in this study. Patient recruitment criteria included:

1. A previous IVF failure despite the transfer of at least 2 best embryos selected from a cohort of good quality embryos.

2. Normal appearance of endometrial cavity in the office hysteroscopic examination, in the early to mid luteal phase.

3. Normal histologic evaluation of the endometrium in the light microscopy examination (those with diagnosis of polyps, endometritis or proliferative endometrium were excluded).

4. Patinets who had luteal phase deficiency was excluded.

5. No use of oral contraceptives in the biopsy cycle.

6. No history of any systemic disease or presence of pelvic inflammatory disease in the last six months.

7. Normal chromosomal analysis.

Routine thrombophilia is not included in all patients, but a routine screening panel for all IVF patients including activated protein C resistance, protein C and S levels and anti-thrombin III levels was performed in order to take precautions to prevent hypercoagulation in a potential ovarian hyperstimulation syndrome.

GROUPS

Group I (n=13) patients were formed from patients who did not conceive in the subsequent IVF attempt, while Group II patients (n=12) were formed from those patients who conceived in their subsequent IVF attempts.

ENDOMETRIAL BIOPSY

Endometrial biopsies were taken between days 17-21 of their natural cycle (previous to IVF treatment) from fundal posterior wall with a hysteroscopic grasper during office hysteroscopy. Office hysteroscopy (Karl Storz, Totlingen, Germany) was

performed as described by Bettochi.[11] The biopsy specimen was immediately immersed and fixed within a 10% formaldehyde solution. After then, specimens were dehydrated accordingly within alcohol, aceton and xylene to prepare parafine blocks. Then, 2 mm sections were sliced from those blocks, stained with hematoxyline and eosine to be examined with light microscopy (Olympus BX-51, Japan). Endometrial dating was assessed using Noyes' criteria. The pathologist assessing the samples was blinded to the treatment groups.

Immunohistochemistry Analysis: Immunostaining was performed on 3-μm thick sections, following deparaffination and rehydration, Endogenous peroxidase activity was blocked with 1% H_2O_2 methanol, washed in Tris-buffered saline(TBS)(15min). The sections were then incubated with the primary monoclonal antibody against Gd-A (mouse monoclonal IgG1, Abcam, Cambridge, England) for 1 hour, biotinylated IgG(15 min), and the avidin-biotin peroxidase complex (ABC) (15 min). Between each of the steps the sections were washed three times in TBS. The reaction was visualized using 3-amino, 9 ethyl carbazole (AEC) chromogen for 15 min and stopped in tap water. After counterstaining with hematoxylin, the slides were dehydrated, coverslipped, and examined using an Olympus BX51 microscope.

Immunoreactivity was evaluated independently by two observers. Staining reactions were assessed semi-quantitatively using the H-Score method. For each section, the intensity of the Gd-A staining was assessed separately in stroma, glandular epithelium and luminal epithelium in 5 distinct high magnification areas (×400 objective), (0=no labelling, 1+=weak, 2+=moderate and 3+=strong labelling). The values in percentages were added together using the following formula: H Score=[(% at 0)×0]+[(% at 1+)×1]+[(% at 2+)×2]+[(% at 3+)×3], and the results were set to an H-Score range (Figure 1, 2).

STATISTICAL METHODS

Statistical analysis of the data was performed using SPSS for Windows v. 11.0 statistical package program (SPSS, Chicago, IL). The Shapiro–Wilk test was used to test for normal distribution of the continuous data. Student's t test, Mann–Whitney test, Pearson's Chi-square test or Fisher's exact test, and ROC Curves were used, where appropriate. Statistical significance was present if P was <0.05.

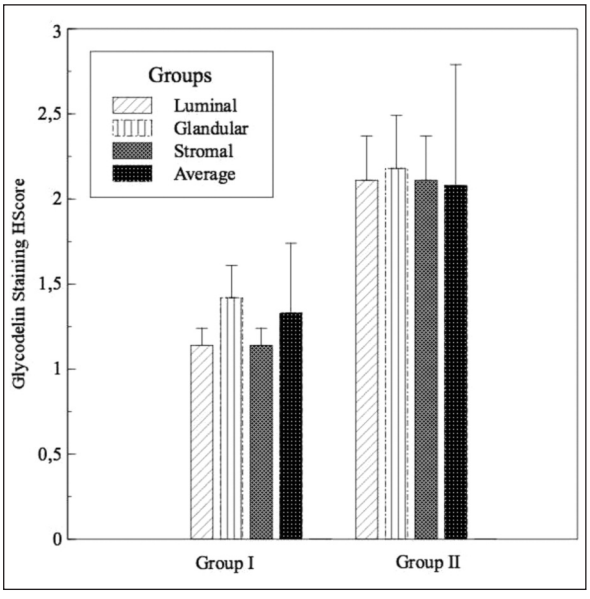

FIGURE 1: Glycodelin staining HScore of Luminal and glandular epithelium and stromal cell.

Interobserver and intraobserver variability was assessed with Reliability Analysis. If intraclass correlation was > 0.80, it was accepted to be statistically significant. For both luminal and glandular epithelium, interobserver and intraobserver variations were 0.99 and 0.99, respectively. For stroma, interobserver and intraobserver variations were 0.96 and 0.97, respectively.

RESULTS

The demographic data of the groups were similar (Table 1). The cycle days of sampling (18.0±2.3 vs. 18.1±2.1) and dating day according to Noyes' criteria (18.0±0.9 vs 18.1±2.9) were similar in Group I and II, respectively.

Total gonadotropin dose, estradiol levels and endometrial thickness at the day of hCG, follicle numbers >13 mm, oocytes retrieved, number and quality of embryos transferred were also similar among the groups (Table 2).

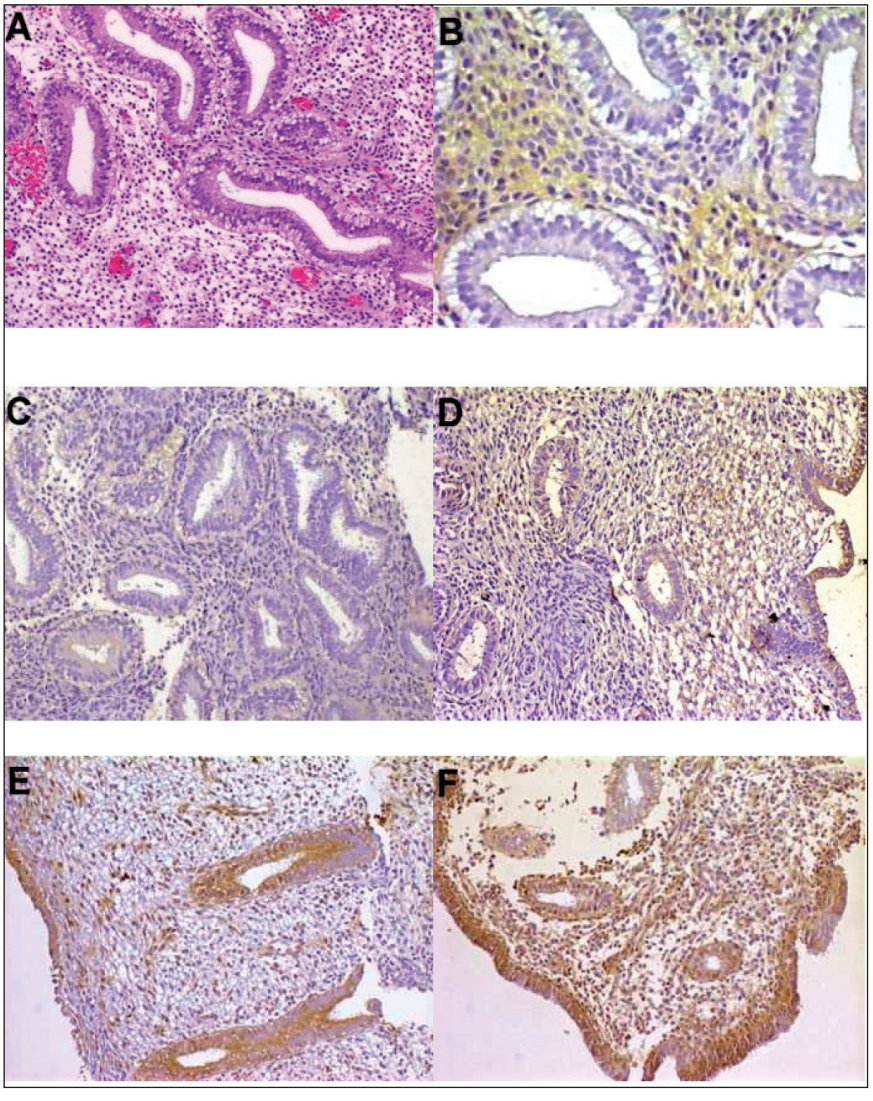

FIGURE 2: A: H&E Section: Endometrium in secretory phase (x20) biopsy day 20
B: Negative glandular epithelium and weak stromal Gd-A expression (x40) Glanduler Epitel HSCORE: 100, Stroma HSCORE: 164, (biopsy day 20)
C: Control, Negative Gd-A expression (x20) Luminal Epitel HSCORE: 100, Glanduler Epitel HSCORE: 103, Stroma HSCORE: 121 (biopsy day 18)
D: Weak Gd-A expression (x20) Luminal Epitel HSCORE: 160, Glanduler Epitel HSCORE: 150, Stoma HSCORE: 130 (biopsy day 16)
E: Moderate Gd-A expression (x20) Luminal Epitel HSCORE: 300, Glanduler E HSCORE: 300, Stroma HSCORE: 300 (biopsy day 18)
F: Strong Gd-A expression (x20) Luminal Epitel HSCORE: 400, Glanduler Epitel HSCORE: 400, Stroma HSCORE: 291 (biopsy day 16)

Gd-A staining was significantly lower in in Group I compared to Group II in the lumens of glands (114.0±32.7 vs. 210.9±84.6, P=0.004) and within the glands (141, .5±68.2 vs. 218.3±105.9, P=0.040) and, there was a trend for statistical significance in the stroma of the glands (138.4±50.9 vs. 186.7±66.3, P=0.05) (Table 3). Receiver operating characteristics (ROC) curves showed statistical significance in luminal (P=0,002) and glandular (p=0,053) H-Score, but best predictor was found to be luminal H-Score (Table 4). When male factor

TABLE 1: Demographic variables of the patients (mean±SD).

	Group 1 (n=13)	Group 2 (n=12)	P
Age (years)	35.2±4.6	32.4±4.8	0.148
BMI (kg/m^2)	23.9±2.8	25.1±2.5	0.267
Day 3 FSH (mIU/ml)	6.4±3.3	6.5±2.3	0.947
Antral follicle count	7.7±5.4	8.3±4.1	0.742
Infertility duration (months)	12.5±6.2	9.1±4.9	0.147
Type of infertility (n,%)			1.0
Female & unexplained factor	8 (61.5)	7 (58.3)	
Male factor	5 (38.5)	5 (41.7)	

TABLE 2: Controlled ovarian hyperstimulation, ICSI and embryo transfer characteristics of the groups.

	Group 1 (n=13)	Group 2 (n=12)	P
GnRH-agonist	11	10	1
GnRH-antagonist	2	2	
Total gonadotropin dose (IU)	3141.3±1151.0	2613.5±982.9	0.232
Estradiol plasma level on the day of hCG (mIU/ml)	1226.2±626.8	1606.3±1042.1	0.310
Endometrial thickness on the day of hCG (mm)	9.4±1.6	9.7±1.4	0.665
Total MII oocytes (n)	6.5±4.1	7.4±2.8	0.532
Transferred embryos	2.7±0.8	3.0±0.7	0.313
Number of transferred embryos of grade 1	2.3±0.8	2.8±0.8	0.081
Easy transfer	9 (75.0%)	9 (69.2%)	

TABLE 3: Glycodelin staining HScore of groups.

HScore	Group I	Group II	P
Luminal epithelium	1.14±0.33	2.11+0.85	0.004
Glandular epithelium	1.42+0.68	2.18+1.05	0.04
Stromal epithelium	1.38+0.51	1.87+0.66	0.52
Average	1.33+0.41	2.08+0.71	0.005

TABLE 4: Area under the curve comparisons.

HScore	Area	SE	p	95% CI
Luminal staining	0.891	0.076	0.002	0.743-1.000
Glandular staining	0.750	0.111	0.053	0.533-0.967
Stromal staining	0.727	0.114	0.078	0.503-0.951
Average	0.814	0.095	0.015	0.628-0.999

was the indication for IVF, Gd-A staining was similar between the groups. However, when the indication was female factor or unexplained infertility, the mean H-Score for Gd-A staining was statistically lower in Group I compared to Group II (Table 5).

DISCUSSION

This study is evaluating the Gd-A expression in lumen of glands (luminal epithelia), within the gland (glandular epithelia), and finally in the stroma of the glands. According to our results, immunohistochemical staining of Gd-A increased in the endometrium of conceiving patient but luminal expression was the best predictor of pregnancy.

The endometrium is primarily composed of luminal and glandular epithelium, and stromal cells. The apical membranous projections of luminal surface epithelium is called pinopode, which is associated with increased glycodelin expression as a marker of the receptive endometrium.[12] Glycodelin was shown to be increased in the receptive phase of the cycle.[13,14] Forced expression of glycodelin in HEC1-B cells significantly increased the attachment of trophoblastic spheroids (i.e., blastocyst surrogate) onto the endometrial epithelial cells.[15] It is not a surprise to find the increased Gd-A expression in luminal epithelium in our study in accordance with pinopode formation.

The explanation for a good correlation between endometrial immunohistochemical expression of GdA and good reproductive outcome could be related to paracrine regulator role in early pregnancy of Gd-A in feto-maternal defense, yet the mechanisms of action are not fully understood. GdA may be involved in early placental development via its modulatory effect on immune and trophoblast cells. GdA inhibits activation and proliferation, and induces apoptosis of T cells. By

TABLE 5: H-Score for Gd-A staining in patients with female factor or unexplained infertility.

	Male Factor			Female and unexplained infertility		
HScore	Group I	Group II	p	Group I	Group II	p
Luminal epithelium	1.00+0.00	2.1+0.83	0.041	1.18+0.36	2.12+0.94	0.023
Glandular epithelium	1.52+0.74	2.20+1.00	0.258	1.35+0.68	2.17+1.18	0.117
Stromal epithelium	1.58+0.72	1.96+0.82	0.458	1.26+0.32	1.80+0.59	0.044
Average	1.44+0.53	2.09+0.81	0.176	1.26+0.33	2.07+0.69	0.022

selectively inducing Th1 cell death, GdA may shift the Th1/Th2 ratio at the feto-maternal interface. This is also achieved indirectly through enhanced expression of Fas in the Th1 cells, thus making them vulnerable to cell death through Fas ligand expressed on trophoblast, endometrial, and activated T helper cells. GdA also promotes secretion of the Th2 cytokines IL-6 and IL-13 from NK cells, and induces immunological tolerance of dendritic cells and apoptosis of monocytes. In addition, specific glycosylation with α2-6 sialic-acid-rich glican of GdA is a prerequisite for the significant immunosupressive activity of GdA. The reduction in α2-6 sialylation of GdA, as in gestational diabetes, is associated with impairment of its T cell apoptosis-inducing activities and its role as a paracrine regulator in early pregnancy.[16]

The studies investigating the effectiveness of GdA as an implantation marker have various conflicting results either by using endometrial flushing fluid (EFF) or plasma Gd-A level.[10] The explanations for those are substantial overlapping of normal and pathological state for plasma level, and lacking of standardized conditions of sampling for EFF. In especially, the endometrial flushing is an inconsistent procedure and the variations in Gd-A measurements could be due to differences in the sampling procedure. The reliability of semi-quantitative immunohistochemical staining of Gd-A in endometrial samples is superior as used as in this study. But we are thinking molecular techniques to determine the amount of Gd-A in the tissues is better than immunhistochemical staining for obtain more precise and quantitative data.

Recently, researchers have focused on assessment of the endometrial receptivity as a major key point in the implantation process for the success of IVF. Limited number of studies evaluating those factors is generally small sample sized and retrospectively designed. The modest aim of the current pilot study was to identify a group of patients for Gd-A expression in an IVF program for a future prospectively designed study with a larger group.

Conflict of interest: Authors declare that there have no conflict of interest.

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