



Ovarian drilling down-regulates endometrial nuclear factor- κ B p65 expression in women with PCOS: A prospective case-control study

Ovaryen drilling PKOS hastalarında endometrial nükleer faktör κ B p65 ekspresyonunu downregüle eder: Prospektif olgu kontrol çalışması

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Abstract

Objective: To investigate the impact of laparoscopic ovarian drilling (LOD) on the expression of endometrial NF κ B p65 (Rel A) in women with clomiphene-resistant polycystic ovary syndrome (PCOS).

Materials and Methods: The study group comprised 25 normal-weight women with PCOS undergoing LOD and 14 control women without PCOS. Endometrial NF- κ B p65 levels evaluated before and after LOD following immunohistochemical staining. The semiquantitative method was used to evaluate the intensity of NF- κ B p65 levels. NF- κ B p65 was found to higher in the endometrium of patients with PCOS compared to controls. LOD leads to significant down-regulation in endometrial NF- κ B p65 expression. NF- κ B p65 expression of PCOS and fertile control were similar after LOD. After LOD, H-score values decreased approximately 3-fold. The H-score of the control subjects was lower than the preoperative and postoperative H-score values of the control women with ovarian cyst.

Results: Expression of endometrial NF- κ B p65 did not change following ovarian cystectomy. The laterality of the ovarian cyst did not cause any change in preoperative H-score values.

Conclusion: By downregulating the endometrial NF- κ B p65 expression LOD improved physiological inflammation in women with PCOS.

Keywords: PCOS, ovarian drilling, clomiphene, nuclear factor κ B, endometrial inflammation

Öz

Amaç: Bu çalışmanın amacı laparoskopik ovaryen drilling (LOD) işleminin klomifen sitrat rezistansı olan polikistik over sendromlu (PKOS) hastalarda endometriyal NF κ B p65 (Rel A) ekspresyon düzeylerini etkileyip etkilemediğinin araştırılmasıdır.

Gereç ve Yöntemler: Çalışma grubu LOD yapılan 25 tane normal kiloya sahip PKOS'li hastadan oluşmaktadır. Kontrol grubunda ise 7 tane PKOS olmayan sağlıklı fertil kadın ve 7 tane de benin kisti olan ancak endometriyomasi olmayan toplam 14 hasta mevcuttur. Endometriyal NF- κ B p65 ekspresyonu LOD öncesi ve sonrası mid-sekretuar fazda ölçülmüştür. Endometriyal örnekler immünohistokimyasal yöntemlerle boyanmıştır. Endometriyal NF- κ B p65 ekspresyonu H-skor metodu ile değerlendirilmiştir.

PRECIS: In this paper, on the basis of our observations by down regulating the endometrial NF- κ B p65 expression laparoscopic ovarian drilling (LOD) improve physiological inflammation and receptivity in women with clomiphene-resistant PCOS.

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Bulgular: Endometriyal NF- κ B p65 ekspresyonu PKOS olan hastalarda LOD öncesi fertil hastalardan daha yüksek bulunmuştur. PKOS'li hastalarda LOD sonrası NF- κ B p65 ekspresyonu belirgin olarak azalmıştır. LOD sonrası PKOS grubundaki hastalar ile fertil grupta NF- κ B p65 ekspresyonu benzer bulunmuştur. LOD sonrası H-skoru 3 kat azalmıştır. Fertil grubun H-skoru pre-op ve post-op dönemde over kistli hasta grubunun H-skorundan düşük bulunmuştur. Yapılan benin kistektomi operasyonları sonrası endometrial NF- κ B p65 ekspresyonunda belirgin değişiklik gösterilmemiştir. Ovaryen kist varlığı pre-operatif H-skor değerlerini etkilememektedir.

Sonuç: LOD, klomifen sitrat rezistansı olan PKOS'li hastalarda endometrial NF- κ B p65 ekspresyonunu azaltarak fizyolojik enflamasyonu ve reseptiviteyi iyileştirmektedir.

Anahtar Kelimeler: PKOS, ovaryen drilling, klomifen sitrat, nükleer faktör κ B, endometrial enflamasyon

Introduction

The physiological amount of inflammation is needed for the blastocyst to be held securely in the endometrium. Inflammation at the pathological level might block attachment and invasion of a blastocyst^(1,2). Pathological inflammation in endometrial tissue may lead to failed decidualization and implantation. Clear scientific data on pathological inflammation preventing implantation come from patients using intrauterine devices^(3,4). In addition to local endometrial pathology diseases located in extra-endometrial areas such as ovaries or fallopian tubes may adversely affect the endometrium receptive status^(1,5). In support of this hypothesis, in the presence of endometrioma or hydrosalpinx pathological inflammation in the endometrium is significantly increased. The reduction of inflammation following salpingectomy or endometrioma cystectomy further confirms the effects of extra-endometrial diseases on endometrial inflammation^(1,6).

In addition to anovulation-failed receptivity due to increased endometrial inflammation may further complicate the implantation in subfertile subjects due to polycystic ovary syndrome (PCOS)⁽⁷⁾. Concordantly, it has been reported that PCOS-related inflammation may cause expression of both steroid hormone receptor and receptivity genes^(8,9). In line with this, the expression of homeobox genes, one of the most important receptivity molecules, decreased in PCOS⁽⁹⁾. Chronic inflammation is one of the most important factors leading to subfertility in PCOS⁽¹⁰⁾. Increased levels of NF- κ B p65, an intracellular marker of inflammation, have been shown in women with PCOS⁽⁷⁾. NF- κ B is a molecule that contains homo and heterodimers in its structure consisting of five different subunits: p50/p105, p52/p100, p65, c-Rel, and RelB. They are bound to its inhibitory protein I κ B α and block the activation of NF- κ B. Following any stimulus, I κ B α is phosphorylated and the NF- κ B is released. NF- κ B dimers migrate to the nucleus where they activate many genes related to inflammation^(1,11).

Laparoscopic ovarian drilling (LOD) is a treatment option in the case of clomiphene-resistant women with PCOS to increase ovulation and pregnancy rates⁽⁹⁾. The increase in the pregnancy rates following LOD was attributed to the increase in ovulation rate or decrease in serum androgen levels^(12,13). Senturk et al.⁽⁹⁾ reported that LOD improves receptivity gene expression in women with PCOS. The increase in homeobox

10 and 11 mRNA after LOD, made us think that LOD may also improve the decreased implantation rates in patients with PCOS by regulating endometrial inflammation. To clarify our prediction we decided to determine NF- κ B p65 expression in the endometrial tissues obtained from women with PCOS. We, therefore, attempted to investigate the effect of LOD on endometrial NF- κ B p65 of infertile women with clomiphene-resistant PCOS.

Materials and Methods

Power analysis of the study was performed with Mann-Whitney U test with the effect size of 0.50% and power of 80%. Type 1 error was noted as 5%. In this case-controlled study endometrial NF- κ B expression was evaluated during the mid-luteal phase from 25 infertile women with clomiphene-resistant PCOS. Patients were diagnosed with PCOS based on the revised Rotterdam criteria. Detail criteria can be found elsewhere. Women who do not respond to clomiphene treatment were accepted as clomiphene-resistant⁽⁹⁾. Failure to achieve ovulation with clomiphene, followed by letrozole, led to the decision of laparoscopy (L/S). Seven infertile women ovarian cysts and seven fertile women without PCOS undergoing tubal sterilization were recruited as controls. While benign ovarian cysts were unilateral in 4 cases, they were recorded bilaterally in 3 cases. In addition to fertile controls, taking patients with benign ovarian cysts as a control group enabled us to test the possible effects of both the presence and surgery of non-endometriotic ovarian cysts on the endometrium. LOD was performed using a monopolar hook and 3 to 5 injury of 1 to 2 mm in the cortex of ovary^(14,15).

Benign ovarian cysts were removed surgically in control participants. Fertile participants underwent endometrial sampling during tubal sterilization. Women with PCOS were subjected to progesterone withdrawal to determine their secretory phases. Noyes criteria were also taken into consideration in endometrial specimens. Serum progesterone levels were measured in each case to determine ovulation. Venous blood was taken from PCOS and control subjects for biochemical and hormonal analysis. Homeostasis model assessment-insulin resistance index (HOMA-IR) was used to determine insulin resistance. Women with history of endometrioma, hydrosalpinx, chronic inflammatory diseases were excluded. The study was conducted approving by the

Local Ethics Committee (approval number: 10973, date: 10.03.2019).

NF- κ B/p65 Staining

Endometrial samples were taken before drilling with a Pipelle cannula. The second samples were taken 3 months later and embedded in paraffin blocks. The immunostaining was performed using ready to use NF- κ B/p65 antibody. Following washing with PBS, the slides were incubated with a peroxidase kit. The slides were developed in DAB, counterstained with hematoxylin. To determine the expression intensity of NF- κ B p65, the H-score was used. $H\text{-score} = \sum P_i (i+1)$, where P_i is the percentage of stained cells in each intensity category (0-100%), and i is the intensity indicating weak ($i=1$), moderate ($i=2$) or strong staining ($i=3$).

Statistical Analysis

Statistical analyses were performed with the SPSS 20.0 software. Data were shown as mean $\pm$ standard deviation values or percentages. Percentages of demographic findings were compared using the Paired t-test. A $p < 0.05$ was considered statistically significant. Kolmogorov-Smirnov test was used for normality of data. While continuous variables were analyzed by using the Mann-Whitney U test Pearson chi-square test was used for categorical data.

Results

Table 1 shows the demographic characteristics of both groups of participants. Fertile controls had no evidence of clinical and ultrasonographical manifestations of PCOS. The average age of the fertile group was recorded as higher than both PCOS and ovarian cyst groups. While the mean ages of the patients with PCOS and ovarian cyst groups were similar, the body

mass index (BMI) was found to be higher in patients with PCOS. BMI of PCOS and control were found similar. BMI of subjects with PCOS was higher than the BMI of women with ovarian cysts. In addition to fasting insulin and total testosterone levels, HOMA-IR levels were significantly higher than those in the fertile control and ovarian cyst group. No difference was found between the three groups in terms of blood glucose values.

Expression levels of NF- κ B p65 were higher in women with PCOS compared to controls. Likewise, endometrial NF- κ B p65 expression was higher in patients with PCOS before LOD compared to women with benign ovarian cysts. The LOD of PCOS ovaries decreased the endometrial NF- κ B p65 expression to the levels of fertile control subjects. Endometrial NF- κ B p65 did not change significantly after ovarian cystectomy. A trend toward decreased endometrial NF- κ B p65 expression was found after ovarian cystectomy compared with the preoperative values. H-score values of endometrial NF- κ B p65 in the fertile group were significantly lower than those of women with PCOS before LOD. The pre-LOD H-score values were approximately twice higher than the H-score values of the ovarian cyst group. Following LOD, H-score values of patients with PCOS and fertile cases were found to be similar. After LOD, H-score values decreased approximately 3 times. The H-score value of the fertile group was significantly lower than the preoperative and postoperative H-score values of the ovarian cyst group. The laterality of the ovarian cyst did not cause any change in preoperative H-score values. Likewise, the fact that the ovarian cyst was unilateral or bilateral did not affect the change in H-score values due to surgery (Table 2). The immunoreactivity of NF- κ B p65 was detected in the cytoplasm of luminal and glandular cells (Figure 1).

Table 1. Demographic and hormonal features of each group of participant

	I-PCOS (n=25)	II-Ovarian cyst (n=7)	III-Fertile control (n=7)
Age	26.9±8.39	27.4±4.30	29.2±4.50
Infertility duration (years)	3.16±1.01	2.81±0.31	-
Cyst size (mm)	-	6.12±0.16	-
Laterality	Bilateral	Unilateral in 4, bilateral in 3 cases	-
BMI (kg/m²)	26.4±1.02*	24.4±4.04	26.7±3.35
Total testosterone (ng/dL)	74.0±1.23*	34.3±4.59	35.9±8.19
HOMA-IR	4.54±1.60*	2.60±1.29	2.77±1.02
Fasting insulin (Mu/mL)	18.1±0.11*	10.3±1.22	11.0±0.03
Fasting glucose (mg/dL)	84.1±1.22	81.4±3.45	86.3±0.50
P-value			
I vs II	BMI: p<0.03, testosterone: p<0.01, HOMA-IR: p<0.03, insulin: p<0.01.		
I vs III	Age: p<0.023, testosterone: p<0.001, insulin: p<0.01		
BMI: Body mass index, HOMA: Homeostatic model assessment, IR: Insulin resistance, PCOS: Polycystic ovary syndrome			

Discussion

In this study, we showed that pathological endometrial inflammation before LOD in clomiphene-resistant PCOS cases increased significantly compared to non-PCOS controls. The LOD of PCOS ovaries decreased the endometrial NF- κ B p65 expression to the levels of fertile control subjects. Endometrial NF- κ B p65 did not change significantly after ovarian cystectomy. Providing normalization in endometrial inflammation following LOD is an important finding that supports the view that a cause of subfertility in PCOS is abnormal endometrial inflammation. Many hormone values of our patients returned to normal after LOD. The decrease in HOMA-IR and androgen values after LOD may be the reason for the decrease in endometrial NF- κ B p65 levels. As a result, by performing ovarian drilling in

PCOS cases we can improve hormonal values and NF- κ B p65 expression close to natural cycles by achieving a reduction in ovarian volume, serum testosterone, and androstenedione levels. In a recent study conducted in clomiphene-resisting PCOS cases, it has been shown that ovarian drilling increases the expression of endometrial receptivity genes⁽⁹⁾. LOD does not require cycle monitoring, is performed once, is inexpensive and has no risk of hyperstimulation, making it an alternative approach to medical treatments. In addition to all these positive effects, it has disadvantages such as surgical complications and the risk of periovarian adhesion.

The pathophysiology of PCOS is examined under four interacting topics such as insulin resistance, hyperandrogenemia, and chronic low-grade inflammation. While clinical and laboratory findings of systemic inflammation are observed in some of the

Table 2. Pre-drilling and post-drilling H-score values of NF kappa B/65 (RelA) expression in PCOS and control groups

Groups	H-score of NF kappa B/65 (RelA) expression		p-value
	Preoperative	Postoperative	
PCOS (n=25)	0.33 $\pm$ 1.27	0.11 $\pm$ 2.32	<0.002
Unilateral ovarian cyst (n=4)	0.18 $\pm$ 1.29	0.10 $\pm$ 1.27	0.069
Bilateral ovarian cyst (n=3)	0.21 $\pm$ 0.12	0.19 $\pm$ 0.34	0.080
Fertile control (n=7)	0.09 $\pm$ 4.78		-

PCOS: Polycystic ovarian syndrome

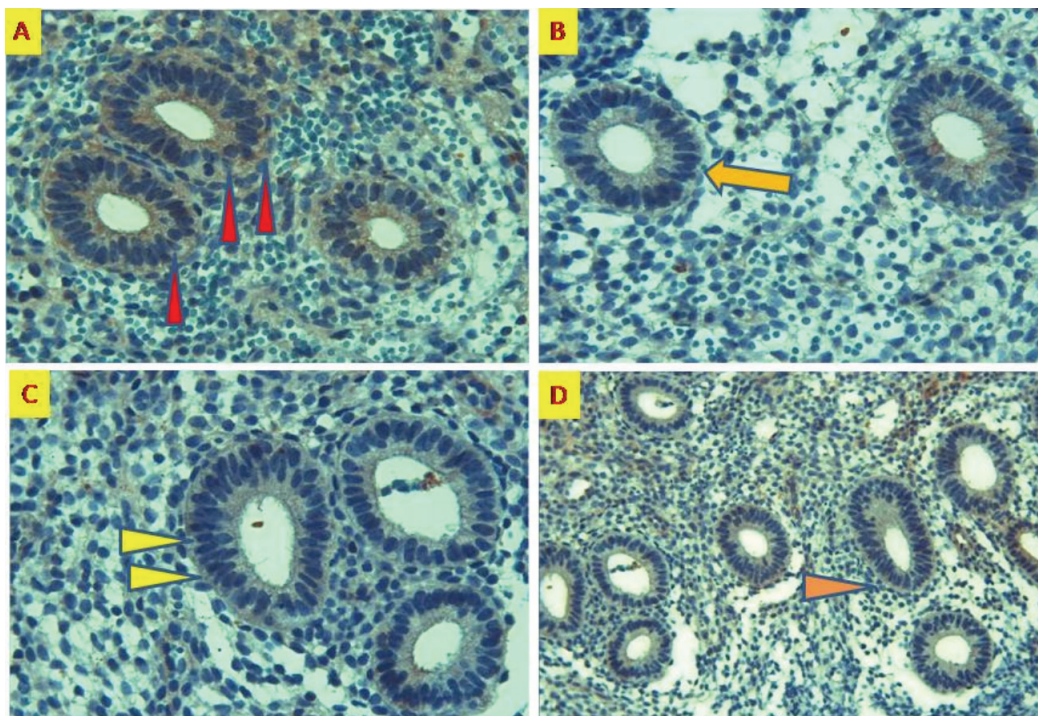


Figure 1. High endometrial NF- κ B p65 immunoreactivity in women with PCOS localized in luminal and glandular epithelium before LOD (A, red arrowheads, X20). Significantly decreased NF- κ B p65 expression after LOD (B, arrow, X20). Normal endometrial NF- κ B p65 expression in ovarian cyst group before cystectomy (C, arrowheads X20). Insignificant decline in NF- κ B p65 expression following cystectomy (D, arrowhead X10)

PCOS: Polycystic ovarian syndrome, LOD: Laparoscopic ovarian drilling

PCOS cases, there is no evidence of the presence of inflammation in some of them. Adipose tissue of patients with PCOS causes pathological changes in insulin sensitivity, lipid metabolism, reproductive system functions, and inflammation through adipokines and proinflammatory cytokines such as hs-C-reactive protein, interleukin-6, and tumor necrosis factor α , adiponectin, visfatin, and omentin^(16,17). Any systemic inflammatory condition located in the reproductive organs may affect the endometrium and then its receptive capacity^(1,3). In line with this, the presence of PCOS, hydrosalpinx, endometriosis, or endometrioma affects the endometrial inflammation irrespective of their location^(1,5,9). Since PCOS is a chronic and systemic inflammatory disease, the endometrium can be negatively affected by this process. Although many oocytes are collected in in vitro fertilization-embryo transfer cycles implantation rates are still relatively low in women with PCOS suggesting failed receptivity or deviation from physiological inflammation. NF- κ B is the most studied molecule in the evaluation of endometrial inflammation. A recent study by Koc et al.⁽⁷⁾ demonstrated that expression levels of endometrial NF- κ B p65 increased in normal and overweight women with PCOS suggesting pathological inflammation. Nevertheless, studies investigating the effect of medical agents or ovarian surgery on PCOS-related pathological endometrial inflammation are lacking. Likewise, the role of LOD in endometrial NF- κ B expression in PCOS-related subfertility has not yet been elucidated yet. Our findings are important in terms of the first demonstration of increased NF- κ B p65 expression in infertile women with clomiphene-resistant PCOS. We clearly showed that expression levels of endometrial NF- κ B p65 were higher in infertile patients with PCOS before LOD compared with healthy fertile controls. Significantly decreased NF- κ B p65 expressions were detected in endometrial samples obtained three months after LOD.

LOD is a minimally invasive surgical procedure preferred in patients with PCOS who are resistant to clomiphene citrate administration, have BMI less than 30 kg/m² and LH levels above 10 IU/L. In infertile PCOS cases, a proper LOD normalizes impaired ovarian morphology, as well as endocrine properties^(12,13,18). A recent study demonstrated that endometrial receptivity genes were shown to be downregulated in clomiphene-resistant PCOS cases⁽⁹⁾. They also reported that LOD improves the expression of receptive genes. Although they did not evaluate the endometrial inflammation following LOD, they suggested that one of the most important causes of a decrease in receptivity molecules is pathological endometrial inflammation. In another work of the same author, it has been reported that NF- κ B expression decreased significantly after endometrioma cystectomy⁽¹⁾. When these results and our findings are evaluated together, we conclude that the endometrium can be indirectly affected in the presence of remote tissue disorders such as endometrioma or systemic diseases such as PCOS. Furthermore, because of the removal of the diseases, the indirect changes on the

endometrium disappear. The decrease in endometrial NF- κ B p65 expression after LOD in clomiphene-resistant PCOS cases is the biggest supporter of these claims. However, the absence of changes in endometrial inflammation in cases of a non-endometriotic benign cyst located in the ovary suggests a special communication between the ovarian tissue and the endometrium in PCOS cases.

Increased endometrial NF- κ B p65 expression may lead to PCOS-associated subfertility and LOD restores pathological inflammatory events. We demonstrated for the first time that LOD improved the pathological endometrial inflammation in infertile women with clomiphene-resistant PCOS. We do not know the basic mechanism of the reduction of inflammation after LOD. However, it is possible to make some assumptions from the results obtained from previous studies. Endometrial inflammation and receptivity increase after salpingectomy is one of the best examples⁽⁶⁾. A significant reduction in the expression of endometrial NF- κ B p65 after endometrioma resection is also a supportive example⁽¹⁾. Similar to these two examples, LOD may have regulated inflammation on the endometrium by causing some morphological, metabolic and hormonal changes in the ovarian tissue. Since LOD does not have a direct effect on the endometrium, the results we obtained should depend on the change in ovarian tissue. Since inflammation is more detected in women with PCOS decrease in ovarian androgen production, stromal thickness and improvement of insulin resistance after LOD may indirectly normalize endometrial NF- κ B p65 expression. Whatever the actual mechanism, the morphological and hormonal changes in the ovary after LOD affect the endometrium positively and correct the pathological inflammation. A reason for the improvement in fertility outcome after LOD may be the decrease of NF- κ B p65 levels in the endometrium. Studies comparing medical treatment with LOD will further strengthen our knowledge of this issue.

Ethics

Ethics Committee Approval: The study was performed approving by the Local Ethics Committee (approval number: 10973, date: 10.03.2019).

Informed Consent: Inform consent was obtained.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: N.D.G., K.G., A.Y., K.C., Ş.H., Design: N.D.G., K.G., A.Y., K.C., Ş.H., Data Collection or Processing: N.D.G., K.G., A.Y., K.C., Ş.H., Analysis or Interpretation: N.D.G., K.G., A.Y., K.C., Ş.H., Literature Search: N.D.G., K.G., A.Y., K.C., Ş.H., Writing: N.D.G., K.G., A.Y., K.C., Ş.H.

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