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Research

Autologous Platelet-rich Plasma Intrauterine Perfusion Improves the Fertility Outcome by Correcting the Thin Endometrium due to Clomiphene Citrate

Otolog Trombosit Açısından Zengin Plazma İntrauterin Perfüzyonu, Klomifen Sitrat Nedeniyle İnce Endometriyumu Düzelterek Doğurganlık Sonucunu İyileştirir

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ABSTRACT

Objective: The primary aim of this study was to investigate the effects of autologous intrauterine platelet-rich plasma (IU-PRP) infusion during ovulation induction with clomiphene citrate (CC) on endometrial thickness (EMT) and clinical pregnancy in patients with polycystic ovary syndrome (PCOS) and thin endometrium. The secondary outcome was to detect possible transformations in oligomenorrheic cycles after PRP.

Methods: This study was conducted on 35 anovulatory PCOS patients aged between 22 and 29 years who applied for infertility treatment. The patients had a thin endometrium in their past history. EMT 7 mm was considered thin endometrium. The diagnosis of PCOS was made according to the revised Rotterdam criteria. A total of 35 patients were divided into two groups according to whether they received PRP or not. Twenty patients received CC plus PRP treatment, whereas 15 patients received CC treatment alone. Patients in both groups were administered CC at a dose of 100 mg/day for 5 days, starting from the 3rd day of progesterone-related withdrawal bleeding. Follicular development and EMT were recorded using transvaginal ultrasonography. In cases with EMT <7 mm, approximately 0.5-1 mL of autologous PRP was infused with the IUI catheter, four days after CC treatment, i.e., on the ninth day of the cycle. EMT was measured and recorded again 3 and 6 days after PRP. Timed intercourse was recommended for cases with a follicle with a mean diameter of at least 16-18 mm. The biochemical and clinical pregnancy rates of both groups were recorded.

Results: Both groups were similar in terms of participant age and body mass index. All participants in the CC plus PRP group were successfully infused with autologous PRP on the ninth day of the cycle. The serum estradiol, testosterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and LH/FSH ratios of both groups were similar. Biochemical pregnancy and clinical pregnancy rates of the CC plus PRP group were significantly higher than those of the CC alone group ($p<0.03$ and $p<0.02$, respectively). Although clinical pregnancy was detected in 5 individuals in the PRP group (25%), clinical pregnancy was recorded in 2 individuals in the CC alone group (13.3%). There was no significant change in the oligo/anovulatory cycle patterns of patients with and without PRP. EMT values on the sixth (4.96±2.11 mm vs. 4.68±2.47 mm, $p<0.37$) and eighth days were similar between the two groups (5.11±3.10 mm vs. 5.29±3.01 mm, $p<0.51$). Compared with the CC alone group, the EMT values measured both at day 12 (6.34±1.09 mm vs. 5.47±3.90 mm, $p<0.02$) and at day 15 (7.44±2.60 mm vs. 6.23±2.70 mm, $p<0.01$) in the PRP group were found to be significantly higher.

Conclusion: IU-PRP infusion in PCOS patients with thin endometrium who underwent ovulation stimulation with CC significantly increased both EMT and clinical pregnancy rates.

Keywords: PCOS, PRP, autologous, endometrial thickness, clinical pregnancy

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ÖZ

Amaç: Bu çalışmanın temel amacı ince endometriyuma sahip polikistik over sendromu (PKOS) hastalarında ovulasyon indüksiyonu sırasında klomifen sitrat (KS) ile otolog intrauterin trombositten zengin plazma (IU-PRP) infüzyonunun endometriyal kalınlık (EMT) ve klinik gebelik üzerine etkilerini araştırmaktır. İkincil sonuç, PRP sonrası oligomenoreik döngülerdeki olası dönüşümleri tespit etmektir.

Gereç ve Yöntem: Bu çalışma, infertilite tedavisi için başvuran, yaşları 22-29 arasında değişen 35 anovulatuar PKOS hastası üzerinde gerçekleştirildi. Hastaların geçmiş anamnezlerinde ince endometriyum mevcuttu. Endometriyal kalınlığın 7 mm'nin altında olması ince endometriyum olarak kabul edildi. PKOS tanısı revize edilmiş Rotterdam kriterlerine göre konuldu. Toplam 35 hasta PRP alıp almamalarına göre iki gruba ayrıldı. Yirmi hastaya KS artı PRP tedavisi uygulanırken, 15 hastaya yalnızca KS tedavisi uygulandı. Her iki gruptaki hastalara progesterona bağlı çekilme kanamasının 3. gününden itibaren 5 gün süreyle 100 mg/gün dozunda KS verildi. Foliküler gelişim ve endometriyal kalınlık transvajinal ultrasonografi ile kaydedildi. Endometriyal kalınlık <7 mm olan olgulara KS tedavisinden dört gün sonra yani siklusun dokuzuncu gününde IUI kateteri ile yaklaşık 0,5-1 mL otolog PRP infüze edildi. EMT, PRP'den 3 ve 6 gün sonra tekrar ölçüldü ve kaydedildi. Ortalama çapı en az 16-18 mm olan folikül olgularında zamanlı ilişki önerildi. Her iki grubun biyokimyasal ve klinik gebelik oranları kaydedildi.

Bulgular: Her iki grup da katılımcı yaşı ve vücut kitle indeksi açısından benzerdi. KS artı PRP grubundaki tüm katılımcılara döngünün dokuzuncu gününde başarıyla otolog PRP uygulandı. Her iki grubun serum östradiol, testosteron, luteinize edici hormon (LH), folikül stimülan hormon (FSH) ve LH/FSH oranları benzerdi. KS artı PRP grubunun biyokimyasal gebelik ve klinik gebelik oranları, yalnızca KS grubuna göre anlamlı derecede yüksekti (sırasıyla p<0,03 ve p<0,02). PRP grubunda 5 kişide (%25) klinik gebelik tespit edilirken, sadece KS grubunda 2 kişide (%13,3) klinik gebelik kaydedildi. PRP uygulanan ve uygulanmayan hastaların oligo/anovulatuar siklus paternlerinde anlamlı bir değişiklik olmadı. Altıncı gün (4,96±2,11 mm vs. 4,68±2,47 mm, p<0,37) ve sekizinci gündeki EMT değerleri iki grup arasında benzerdi (5,11±3,10 mm vs. 5,29±3,01 mm, p<0,51). Yalnızca KS grubuyla karşılaştırıldığında, EMT değerleri hem 12. günde (6,34±1,09 mm vs. 5,47±3,90 mm, p<0,02) hem de 15. günde (7,44±2,60 mm vs. 6,23±2,70 mm, p<0,01) PRP grubunda anlamlı olarak yüksek bulunmuştur.

Sonuç: KS ile ovulasyon stimülasyonu uygulanan ince endometriyumlu PKOS hastalarında uygulanan IU-PRP infüzyonu hem endometriyal kalınlığı hem de klinik gebelik oranlarını anlamlı derecede artırmaktadır.

Anahtar Kelimeler: PKOS, PRP, otolog, endometriyal kalınlık, klinik gebelik

INTRODUCTION

Polycystic ovary syndrome (PCOS) is the most common (5-8%) multisystemic endocrine and metabolic disorder in women of reproductive age. Its incidence may be up to 13% according to the population studied (1,2). The presence of at least two of the criteria for oligoovulation and/or anovulation, biochemical or clinical evidence of hyperandrogenism, and polycystic ovarian morphology is sufficient to diagnose PCOS (3). Although most women with PCOS experience oligomenorrhea or amenorrhoea due to anovulatory cycles, both heavy and irregular bleeding can sometimes occur. Although oligo-ovulation is accepted as the main cause of infertility in patients with PCOS, even assisted reproductive techniques do not satisfactorily increase the decreased pregnancy outcomes in patients with PCOS (4). These data suggest that anovulation alone is not responsible for subfertility in PCOS. In connection with the last sentence, increased early pregnancy loss in women with PCOS suggests that parameters such as endometrial dysfunction, obesity, and hyperandrogenemia also affect the fertility status of patients (5).

The PCOS endometrium differs from the healthy endometrium in terms of histomorphology, steroid receptors and coactivators, and receptivity modulators. Therefore, the receptive status of the endometrium is often not suitable for implantation, and endometrial dysfunction in addition to oligoanovulation also contributes to subfertility. In PCOS, progesterone-dependent changes in the endometrium do not occur because of oligo-ovulation or anovulation. Increased androgen-estrogen conversion in peripheral tissue and increased free estradiol and testosterone levels due to hyperinsulinemia may cause unpredictable bleeding in the absence of progesterone. The endometrium of women with PCOS exhibits increased expression of ERα and AR compared with healthy controls (6,7). Differences in receptor expression in PCOS patients may be related to unopposed estrogen elevation. Both circulating portions of estrogen and progesterone and differences in receptor expression prevent the opening of the implantation window in PCOS. On the other hand, the PCOS endometrium fails to produce a physiological response to progesterone, which is an important evidence of progesterone resistance (8). In summary, decreased progesterone levels and receptor expression defects in patients with PCOS lead to the presence of unopposed estrogen, leading to disruption of the normal menstrual cycle and subfertility. Most PCOS patients present with oligo- or amenorrhoea although there is rarely heavy bleeding due to increased estrogen levels.

In the absence of ovulation and progesterone, the endometrium does not undergo secretory transformation and is constantly exposed to estradiol. However, although this rarely leads to excessive bleeding and hyperplasia, in most PCOS cases, menstrual cycle intervals are prolonged and become oligomenorrheic. Although making the cycles ovulatory with medical agents restarts the menstrual cycle,

the picture of amenorrhoea reappears when the treatment is stopped. Platelet-rich plasma (PRP), because of its regenerative potential, is more widely used in reproductive medicine, as it is in every field of medicine (9,10). Although there are studies showing improvement in fertility outcome after the use of intraovarain or intraendometrial PRP in reproductive medicine, most of them are studies with low level of evidence due to both design, PRP standardization, and inhomogeneous participant population (9,10). A thin endometrium is defined as an endometrial thickness (EMT) of 7 mm or less on the day of hCG or embryo transfer (11). The number of studies investigating the effects of PRP application on EMT and fertility outcome in an infertile patient population is quite limited. Although EMT varies in different phenotypes of PCOS, the incidence of a thin endometrium is higher in women with PCOS where hyperandrogenemia is dominant (12). In patients who have ovulation induction with clomiphene, the EMT is further thinned. The primary aim of this study was to investigate the effects of autologous intrauterine PRP (IU-PRP) application during ovulation induction with clomiphene citrate (CC) on EMT and fertility outcomes in patients with PCOS with thin endometrium (<7 mm) resistant to adjuvant treatments. The secondary outcome was to detect possible transformations in oligomenorrheic cycles after PRP.

METHODS

This study was conducted on 35 anovulatory PCOS patients, aged between 22 and 29 years, who applied to our gynecology and obstetrics clinic for infertility treatment. The patients had a history of a thin endometrium in their previous treatment history. EMT 7 mm was considered thin endometrium. The diagnosis of PCOS was made according to the revised Rotterdam criteria. Those who met at least two of the criteria for amenorrhoea/oligomenorrhea with chronic anovulation, clinical and/or biochemical evidence of hyperandrogenism, and ultrasonographic appearance of PCOS were accepted as having PCOS. The diagnosis of anovulation was confirmed by the patient's history and hormonal measurements. From the anamnesis of the patients, it was noted that adjuvant treatments, such as oral or patch-form estrogen supplementation, low-dose aspirin, and vaginal sildenafil, were applied to increase the EMT, but they did not benefit.

It was explained to the patients that PRP treatment is not a routine practice for the treatment of infertility and for increasing the thickness of the endometrium. It was emphasized that there were studies that reported positive results in EMT and fertility outcome after PRP, and studies that reported no benefit. The study was initiated after obtaining permission from the Clinical Research Ethics Committee of Zeynep Kamil Gynecology and Pediatrics Training and Research Hospital (decision no: 14, date: 11.01.2023). In addition, an informed volunteer consent form was obtained from all patients regarding the procedure to be performed. Twenty patients who accepted PRP and 15 patients who did not were divided into two groups and included in the CC treatment protocol. CC (Serophene, Serono, Roma, Italy) was administered to the patients in both groups for 5 days, starting from the 3rd day of progesterone-induced withdrawal bleeding at a dose of 100 mg/day. The patients were called for control on the sixth and eighth days of treatment. Follicular development and EMT were recorded using transvaginal ultrasonography (TV-USG). IU-PRP infusion was planned for cases with EMT <7 mm on the eighth day of follow-up. Endometrial PRP infusion was applied to the patients in the PRP group, four days after the 5-day CC treatment, i.e., on the ninth day of the cycle. In the CC alone group, PRP was not applied. In the TV-USG examination after PRP, both EMT and follicle diameter were measured. Timed intercourse was recommended for cases with a follicle with a mean diameter of at least 16-18 mm. The biochemical and clinical pregnancy rates of both groups were recorded. Clinical pregnancy was defined by the presence of an intrauterine gestational sac confirmed by TV-USG.

Autologous PRP Preparation and Endometrial Infusion

Autologous PRP was prepared from each patient following a two-stage centrifugation using autologous blood. On the 9th day of ovulation stimulation with CC, 15 mL of venous blood was drawn into a syringe containing acid citrate A anticoagulant solution and immediately centrifuged at 300 rpm for 5 min. In the first procedure, red blood cells were removed from the medium. Subsequently, the plasma was centrifuged again at 700 rpm for 17 min and autologous PRP was obtained. Platelet activation was achieved by adding $CaCl_2$ and human thrombin. Activated PRP was finally centrifuged at 3000 rpm for 20 min, and the supernatant was collected. The collected pellets contained erythrocyst- and leukocyte-free PRP. Approximately 0.5-1 mL of PRP was infused with the IUI catheter into the endometrial cavity of the patient in the lithotomy position, accompanied by ultrasonography. No anesthesia or sedation was used during PRP infusion (13). EMT was measured and recorded again 3 and 6 days after PRP infusion.

Statistical Analysis

The data were analyzed using the Statistical Package for Social Sciences software 18.0 for Windows package

software (SPSS). Normality of data was examined using the Kolmogorov-Smirnov test. Mean values were calculated for data showing abnormal distribution and compared with the non-parametric Mann-Whitney U test. Categorical data are described either by number of cases or percentages. Categorical variables were presented as percentage values and compared using the chi-square/Fisher's Exact test. Continuous variables were analyzed using the Mann-Whitney U test. The results are presented as mean ± standard deviation. For all tests, p-value <0.05 was considered statistically significant.

RESULTS

Demographic, hormonal, and reproductive parameters of the CC plus PRP and CC alone groups are presented in Table 1. Both groups were similar in terms of participant age and body mass index. All participants in the CC plus PRP group were successfully infused with autologous PRP on the ninth day of the cycle. No PRP application was applied to the CC alone group. Serum estradiol, testosterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and LH/FSH ratios of both groups were similar. CC doses and administration times were similar in both groups. Biochemical pregnancy and clinical pregnancy rates of the CC plus PRP group were significantly higher than those of the CC alone group (p<0.03 and p<0.02, respectively). Although clinical pregnancy was detected in 5 individuals in the PRP group (25%), clinical pregnancy was recorded in 2 individuals in the CC alone group (13.3%). No significant change was observed in the oligo/anovulatory cycle patterns of patients with and without PRP.

A detailed presentation of the change in the EMT values of the PRP and CC alone groups is shown in Table 2. EMT values on the sixth (4.96±2.11 mm vs. 4.68±2.47 mm, p<0.37) and eighth days were similar between the two groups (5.11±3.10 mm vs. 5.29±3.01 mm, p<0.51). EMT was not evaluated because PRP was performed on the ninth day. Compared with the CC alone group, the EMT values measured both at day 12 (6.34±1.09 mm vs. 5.47±3.90 mm, p<0.02) and at day 15 (7.44±2.60 mm vs. 6.23±2.70 mm, p<0.01) in the PRP group were found to be significantly higher.

DISCUSSION

Implantation in mammals is a very complex process that requires coordinated cross-talk between the blastocyst and endometrium through molecular pathways. Before implantation, the endometrium must be exposed to adequate levels of estrogen and progesterone to make it susceptible to blastocyst invasion. Ovarain sex steroids exert their effects on the endometrial epithelium and decidualized stromal cells through mediator molecules and immune cells. Peripheral blood-derived immune

Table 1. Comparison of demographic and fertility outcome parameters of PCOS patients in CC plus PRP and CC alone groups

Parameters	CC plus PRP	CC alone	p-values*
N	20	15	-
Age (yrs)	26.2±5.43	25.4±4.30	0.462
BMI (kg/m^2)	25.3±5.44	25.8±4.98	0.351
IU-PRP, n (%)	20 (100%)	0 (0)	NA
Total testosterone (ng/dL)	41.7±7.88	43.6±5.01	0.339
Estradiol (pg/mL)	49.3±5.49	50.3±7.20	0.544
LH (mIU/mL)	8.32±2.09	9.13±3.76	0.701
FSH (mIU/mL)	5.67±2.91	5.67±2.10	0.673
LH/FSH ratio	1.46	1.61	0.09
CC dose and duration	100 mg/day, five days	100 mg/day, five days	NA
Endometrila thickness on 6th day of CC treatment	4.96±2.11	4.68±2.47	0.729
PRP time	9th day of the cycle	No PRP	NA
Cycle pattern change after PRP	No (anovulatory)	No (anovulatory)	NA
Biochemical pregnancy, n (%)	6 (30%)	3 (20%)	0.03
Clinical pregnancy, n (%)	5 (25%)	2 (13.3%)	0.02

Data presented as means ± standard deviation. PCOS: Polycystic ovary syndrome, BMI: Body mass index, IU-PRP: Intrauterine platelet rich plasma, FSH: Follicle-stimulating hormone, LH: Luteinizing hormone, CC: Clomiphene citrate, *p<0.05

Table 2. Comparison of endometrial thickness values of CC alone and CC plus PRP groups

CC plus PRP					CC alone (without PRP)				
6th*	8thμ	9th	12th#	15thΣ	6th	8th	9th	12th	15th
4.96±2.11	5.11±3.10	IU-PRP was applied	6.34±1.09 3rd day of PRP	7.44±2.60 6th day of PRP	4.68±2.47	5.29±3.01	No IU-PRP	5.47±3.90	6.23±2.70

*The 6th day EMT value of the PRP group was similar to the CC alone group (p<0.37). μThe 8th day EMT value of the PRP group was similar to the CC alone group (p<0.51). #The 12th day EMT value of the PRP group was significantly higher than the CC alone group (p<0.02). ΣThe 15th day EMT value of PRP group was significantly higher than CC alone group (p<0.01).
CC: Clomiphene citrate, IU-PRP: Intrauterine platelet rich plasma, EMT: Endometrial thickness

cells, leukocytes, and platelets mediate the effects of estrogen and progesterone. These effects are mediated by paracrine mechanisms and prepare the endometrium for implantation by regulating the release of growth factors and cytokines. This multi-molecular pathway results in endometrial decidualization, proliferation in epithelial and stromal cells, increased synthesis of receptivity modulators, and provision of an end-point receptive endometrium (14,15). However, pre-implantation reactions at the histomorphological, molecular, and genomic levels in the endometrium can keep the endometrium in the receptive phase within a period of approximately 6 days. Although the endometrium exhibits these cyclic changes regularly in most women of reproductive age, implantation failure remains the most frequent and serious problem in assisted reproductive techniques (16). Therefore, it is critical to determine whether the endometrium is in the receptive phase before implantation and to choose a treatment approach accordingly. Endometrial receptivity tests developed for this purpose in the last decade have not been routinely used because of their invasive nature and low sensitivity and specificity. EMT measurement with ultrasonography, an old but effective marker, remains the only non-invasive and reproducible method available to evaluate the receptive endometrium.

In the ultrasonographic evaluation of the endometrium, a prediction can be made about endometrial receptivity by evaluating echogenicity and thickness. In addition to the hypoechogenic appearance in sonographic evaluations performed on the day of ovulation or hCG, the detection of an EMT of 7 mm or more strongly supports the presence of a receptive endometrium (17). However, pregnancy has also been reported in hyperechogenic endometriums with a thickness of approximately 4 mm (18). Although the presence of high EMT is not always compatible with receptivity status, the chance of pregnancy is lower in women with thin endometrium. A value of <7 mm, measured on the day of ovulation or hCG, is considered as thin endometrium and means that the success rate for implantation is low (11). PCOS is an endocrine cause of subfertility with ovulatory dysfunction, receptivity defect, and thin endometrial development. Although EMT is normal in some PCOS phenotypes, a thin endometrium is more common, especially in PCOS phenotypes with hyperandrogenemia (12). In particular, in cycles using CC for ovulation induction, EMT is seen to be more thinned (19). Although EMT has been increased with methods such as sildenafil citrate, aspirin, and mechanical endometrial injury, the results are heterogeneous (20). There is no study design using PRP to increase EMT in patients with PCOS using CC for ovulation stimulation. This is the first clinical study investigating the effects of PRP on EMT and fertility outcome in patients with PCOS who could not conceive due to thin endometrium in previous cycles. EMT values of both groups were similar before PRP application. We found that EMT measurements performed 3 and 6 days after IU-PRP application increased significantly. Similarly, both biochemical and clinical pregnancy rates in the PRP group increased significantly compared with the control group. Because the oligo-anovulatory cycle patterns of both groups did not change, we can argue that PRP has a phase-specific effect and has no long-term and clear effect on ovulatory functions.

One of the possible reasons for the significant increase in EMT and clinical pregnancy rates in PCOS patients administered PRP compared with control PCOS patients is the regenerative effects of PRP on the endometrium. PRP is a platelet product that is free from leukocytes and erythrocytes. In addition to many growth factors in PRP, anti-inflammatory and pro-inflammatory molecules create a suitable microenvironment for implantation by regulating decidualization and redox reactions (13,21). In animal studies, treatment of mating-induced endometrial inflammation with PRP infusion supports the immunomodulatory and anti-inflammatory role of PRP (22). The decrease in endometrial development and blood flow due to CC may have been caused by the intense cytokine and growth factor

content of PRP. Increased expression of receptivity genes in PRP-treated animals suggests that a similar effect may occur in patients with PCOS treated with PRP (19,23). The presence of intense interleukin, tumour necrosis factor-α, and interferon-α in PRP may regulate clomiphene-induced endometrial developmental defects and decreased blood flow. The fact that an increase in both EMT and endometrial vascularization was reported in Power Doppler analysis studies performed after PRP is an important evidence of the net effect of PRP on vascular demodulation (24).

In addition to studies reporting improvement in EMT and fertility outcome after IU-autologous PRP infusion (10), there are also studies reporting that PRP has no effect on EMT and implantation (21,25). Although the regenerative effect of PRP has been demonstrated in a murine model of endometrial damage, no human studies have investigated the effect of PRP on receptivity modulators (26). Our study is clinically important in terms of presenting the first clinical data reporting that clomiphene-induced thin endometrial growth is improved with PRP. However, the low number of cases and the application of PRP once is an important handicap. Multiple infusions of autologous PRP may reveal the relationship between EMT and clinical pregnancy rates more clearly. The lack of standardization of the PRP preparation methods may be the main reason for the difference in the results. Applying the buffy coat obtained using only two-stage centrifugation to the endometrial cavity is not a real PRP. Because leukocytes are not removed from the environment in such infusions, we cannot determine whether the effect on the endometrium is due to leukocytes or cytokines. Similarly, the administration of PRP without activation limits its effectiveness. Activated PRP infusion should also be performed quickly and without release of platelet contents. In our study, activated PRP was infused as soon as it was prepared. The fact that the participants were PCOS patients using CC and serial EMT measurements is critical in determining PRP efficacy.

CONCLUSION

In conclusion, autologous IU-PRP increases both EMT and clinical pregnancy rates in patients with PCOS with thin endometrium due to CC. However, the application of IU-PRP did not cause a significant change in the oligo/anovulatory cycle patterns of the patients. We can make a clearer judgment about the effectiveness of PRP in PCOS using case-controlled studies with multiple PRP applications.

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ETHICS

Ethics Committee Approval: The study was initiated after obtaining permission from the Clinical Research Ethics Committee of Zeynep Kamil Gynecology and Pediatrics Training and Research Hospital (decision no: 14, date: 11.01.2023).

Informed Consent: In addition, an informed volunteer consent form was obtained from all patients regarding the procedure to be performed.

Authorship Contributions

Surgical and Medical Practices: R.Ö., E.B., Concept: R.Ö., E.B., M.B.Y., Design: R.Ö., E.B., Data Collection or Processing: R.Ö., E.B., M.B.Y., Analysis or Interpretation: R.Ö., E.B., Literature Search: R.Ö., E.B., M.B.Y., Writing: R.Ö., E.B.

Conflict of Interest: No conflict of interest was declared by the authors.

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